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14. ABSTRACT

During 3-d year, we completed comparing blast load characteristics producing mild through severe TBI of 'composite,' accompanied with head acceleration with primary blast-peak overpressure exposure w/o significant head acceleration. Improved prototype (final version) of cumulative blast sensor and signal conditioning circuit has been produced and tested at Banyan Biomarkers November/December, 2013 and January/February 2013. We were capable of capturing at least 3 consecutive blasts and integrate a cumulative load. Also, we continued to fully characterize brain injury and biomarkers after repeated primary blast exposure. Rats were subjected to a primary blast of 50-53 psi overpressure and total duration of 75 μsec : 3 exposures, with a 45 min to 1 hr recovery at the frontal part of the rat's skull. Portable cumulative blast sensors were placed on the head front and rat spine. We measured blood accumulation of our 'golden standard' GFAP, neuronal UCH-L1 and CNPase, and NSE. To complete a panel, we investigated neuroendocrine peptide Orexin A, Neuropilin-2 and inflammatory sICAM and metalloproteinases. Multiple blasts augmented increased GFAP, UCH-L1, NSE and NRP-2, but not Orexin A, while at 7 days the cumulative effects of multiple blasts were much lower, if any. On the other hand, serum CNPase and sICAM after multiple blasts was significantly augmented vs. single blast both at 1 day and 7 days post exposure. Finally, we found and characterized new signatures of blast injuries: thrombin activity measured by calibrated activated thrombography (CAT), linked to microcirculation disorders following blast exposures.

15. SUBJECT TERMS
Multiple blast exposures; brain injury; 'composite' blast; primary blast; Cumulative Blast and Impulse Exposure Sensing Package (CBI-ESP); GFAP; CNPase; NSEOrexin A; Neuropilin-2 (NRP-2), sICAM, thrombin biomarkers

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1. Cumulative Blast and Impulse Exposure Sensor Package (CBI-ESP) Version 2.0 **USER MANUAL** Hector Gutierrez and Daniel Kirk, Department of Mechanical and Aerospace Engineering, Florida Institute of Technology, Melbourne FL 32901 **January 3rd, 2013**

2. Prima V, Serebruany VL, Svetlov A, Hayes RL and Svetlov SI Impact of Moderate Blast Exposures on Thrombin Biomarkers Assessed by Calibrated Automated Thrombography in Rats J Neurotrauma. 2013 Oct 4. [Epub ahead of print]

3. Tümer, Svetlov SI, Whiddenh M, Kirichenko N, Prima V, Erdos B, Sherman A, Kobeissy F, Yezierski R, Scarpace PJ, Vierck C and Wang K.W. Overpressure blast-wave induced brain injury elevates oxidative stress in the hypothalamus and catecholamine biosynthesis in the rat adrenal medulla. Neuroscience Lett. 2013 Jun 7;544:62-7

4. Kobeissy F, Mondello S, Tumer N, Toklu HZ, Whidden MA, Kirichenko N, Zhang Z, Prima V, Yassin V, Svetlov SI, Wang KKW Assessing Neuro-Systemic & Behavioral Components in the Pathophysiology of Blast-Related Brain Injury. Frontiers in Neurotrauma, 2013 (in press).

5. Adams S, Condrey JA, Tsai HW, Prima V, Svetlov SI, Sumners C, and Davenport PW. Anxiety Produced in Rats by Over-Pressurization Blast Injury. Poster presented at International Society for the Advancement of Respiratory Pyschophysiology held in Leuven, Belgium, September 22-26, 2012.

Introduction.

Objectives of the project are: (i) Develop an experimental framework for reproducing multiple blast wave exposures and recording multiple blasts in an animal model using a prototype sensor device, (ii) define cumulative blast load upon multiple blast exposures and distinguish biomarkers of mild through severe TBI, (iii) identify and characterize biochemical markers of multiple vs. single blast exposures, and formulate blast load injury scale.

In year 1, we determined blast load characteristics producing mild through severe TBI and defined 'composite' and primary blast parameters. Schlieren optics was used to visualize blast wave interaction with experimental animal. The pathological effects of primary blast overpressure were compared with brain injury via 'composite' blast accompanied by strong head acceleration. We assessed neuro-glial injury evaluated by silver staining, and GFAP/CNPase and NSE. Biomarkers of neuro-glial injury GFAP, CNPase and NSE were accumulated in circulation in a particular time-dependent fashion. Sensor package for detecting/recording a cumulative blast exposure was designed and version 1.1 of sensor was produced. **In year 2** of the project we nearly accomplished characterization severe to moderate TBI upon primary blast (peak overpressure) vs. composite blast, (ii) continued developing a cumulative blast detecting/recording module, and (iii) began characterization of biomarkers of TBI in response to multiple vs. single blast exposures. We characterized several systemic/inflammatory, neuroendocrine and growth factor signatures and revealed their relationships and diagnostic value (L-selectin, sICAM, Orexin A, neuropilin-2). Compared to single exposure, multiple blasts augmented increases of GFAP, UCH-L1, NSE and NRP-2, but not Orexin A, at day 1 post-blast, while at 7 days the cumulative effects were much lower, if any. The improved prototype of cumulative blast sensor and signal conditioning circuit were tested at Banyan Biomarkers on 19 October 2012. **In year 3**, improved prototype (final version) of cumulative blast sensor and signal conditioning circuit has been produced and tested at Banyan Biomarkers November/December, 2013 and January/February 2013. We were capable of capturing at least 3 consecutive blasts and integrate a cumulative load. Also, we continued to fully characterize brain injury and biomarkers after repeated primary blast exposure adding and characterizing novel molecular signatures such as metalloproteinases and thrombin generation biomarkers. We found that in contrast to GFAP/UCH-L1, NSE and NRP-2, serum CNPase and sICAM after multiple blasts was significantly augmented vs. single blast both at 1 day and 7 days post exposure.

BODY

In the body section, I present general outline of fully functional v2 of the cumulative blast sensor. The detail pertaining to the sensor are provided in Manual, see Appendix. Also, I present new data on Thrombin biomarker signatures of a single moderate primary blast vs. 'composite' blast. In addition, comparing biomarkers of single and multiple blast exposure, is presented which include sICM as inflammatory signature and CNPase (in depth analysis).

A version 2 of cumulative blast sensor package has been developed and preliminary tested at Banyan Biomarkers using an improved shock tube.

The system is composed of the following components:

1. The data acquisition board (DAQ board)
2. The sensor board
3. Power source (Lithium polymer rechargeable battery)

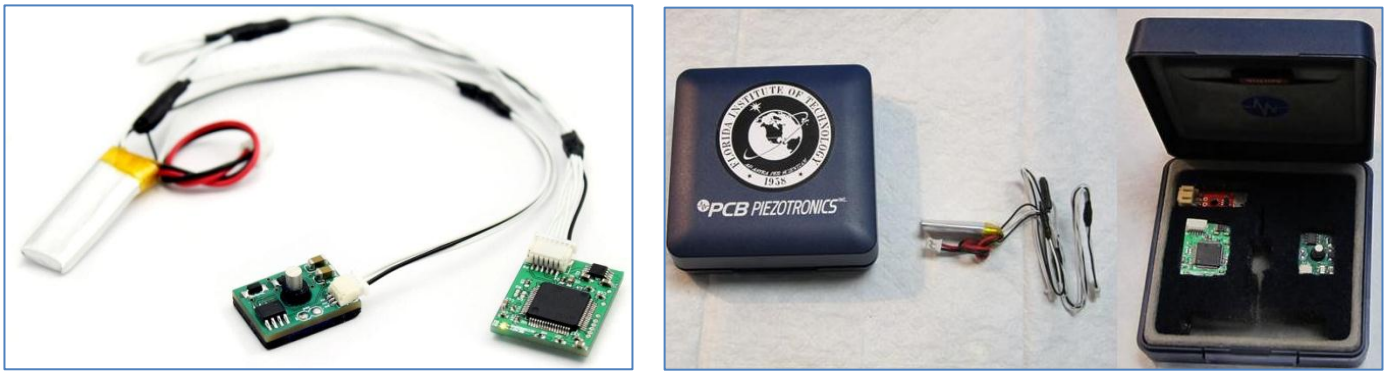

Figure 1: Complete cumulative injury-energy sensing package (CBI-ESP), final delivery

Figure 2 Real size of CBI-ESP sensor and DAQ board

To verify functionality and accuracy of the CBI-ESP v2 system, a comparison between data from the CBI-ESP v2.0 and a standard off- the-shelf high speed blast pressure sensor (PCB-Piezotronics) was conducted using a National Instruments PXI-8331 high speed data acquisition card for data collection.

Both sensors were placed outside the venting cone of the shock tube as shown in Figure 3, (a) and (b). In this configuration, both sensors are expected to read approximately the same pressure event at similar phase, although some minor differences are expected due to reflections from the experimental setup.

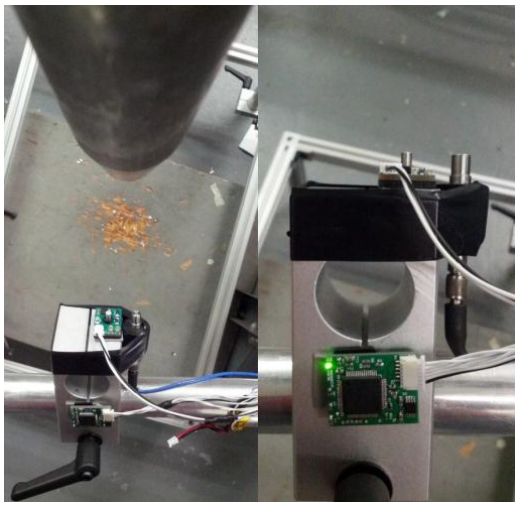

Figure 3 (a): Location of the CBI-ESP sensor board (right) relative to the witness sensor (PCB Piezotronics)

The test was conducted with 1000 psi driver pressure and a driven/driver ratio of 15, using a 0.002 in thick stainless steel shim. The results are shown in **Figure 4.** The standard system was sampled using NI-LabView at 10 Megasamples/sec, and the CBI-ESP v2.0 operated at its native sampling rate of 1 Megasamples/sec.

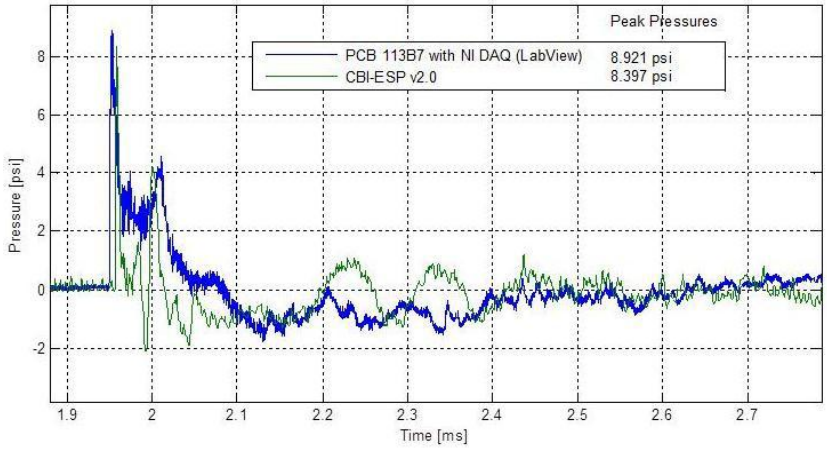

Figure 4 Calibration and validation. Standard blast measurement system (PCB Piezotronics pressure transducer with NI-LabView) compared to the same blast event captured using the CBI-ESP v2.0.

The difference between traces and secondary peak is attributed to slight differences in sensor placement and overpressure wave reflections from two sensors.

Using this package, we performed the series of experiments using repeated blast exposures in rats. We subjected rats combined blast exposure, the first being blast 'on axis' accompanied by head acceleration and two subsequent, with 1 h interval, primary blast OP exposure with negligible or no head acceleration according to the following Table1 .

Table 1 Setup and description of repeated blasts.

Blast OBI rats 11-28-2012_12-05-2012> Protocol #:201004486> Requisition #13054

	ID #	SSB	Received Date	Birth Date	Sex	Treatment	Treatment Date	# Animals	Comments
4	380, 381	SD	11/27/12	09/29/12	Male	Single blast	11/28/12	2; sOBI	Single on-axis blast (#380:)
5	382, 383	SD	11/27/12	09/29/12	Male	Single blast	11/28/12	2; sOBI	Single on-axis blast (#382:)
6	384, 385	SD	11/27/12	09/29/12	Male	Single blast	11/28/12	2; sOBI	Single on-axis blast (#384:)
8	386, 387	SD	11/27/12	09/29/12	Male	Multiple blast	11/29/12	2; mOBI	Single on-axis blast followed by two more off-axis blasts with ~1 h pauses.
9	388, 389	SD	11/27/12	09/29/12	Male	Multiple blast	11/29/12	2; mOBI	Single on-axis blast followed by two more off-axis blasts with ~1 h pauses.
0	390, 391	SD	11/27/12	09/29/12	Male	Multiple blast	11/29/12	2; mOBI	Single on-axis blast followed by two more off-axis blasts with ~1 h pauses.
1	392, 393	SD	11/27/12	09/29/12	Male	No treatment	NA	2, naive	Naïve control
2	394, 395	SD	11/27/12	09/29/12	Male	No treatment	NA	2, naive	Naïve control
3	396, 397	SD	11/27/12	09/29/12	Male	No treatment	NA	2, naive	Naïve control
4	398, 399	SD	11/27/12	09/29/12	Male	Sham treatment	12/05/12	2, sham	Sham control; ~130 dB noise exposure
5	400, 401	SD	11/27/12	09/29/12	Male	Sham treatment	12/05/12	2, sham	Sham control; ~130 dB noise exposure
6	402, 403	SD	11/27/12	09/29/12	Male	Sham treatment	12/05/12	2, sham	Sham control; ~130 dB noise exposure
8	NA	SD	11/27/12	09/29/12	Male	NA	NA	1	Was used in mOBI series to replace #386

Serum and brain tissue was collected and is being analyzed. Cumulative blast load data are being assessed using MatLab software. The ID numbers for blast-exposed animals are as printed on the labels for 1.5 ml serum collection tubes: 6 sOBI animals (IDs: 380-385); 6 mOBI animals (IDs: 386-391); 6 naïve animals (IDs: 392-397); 6 sham animals (IDs: 398-403).

Task 5 through task 7 of **Specific aim 3** are in progress and are slightly modified. Specifically, we omitted measurement of MBP and focused on studies of CNPase as better marker of myelination disorders and NSE as marker of neuronal injury, which reflects also non-CNS involvement, e.g. platelet activation.

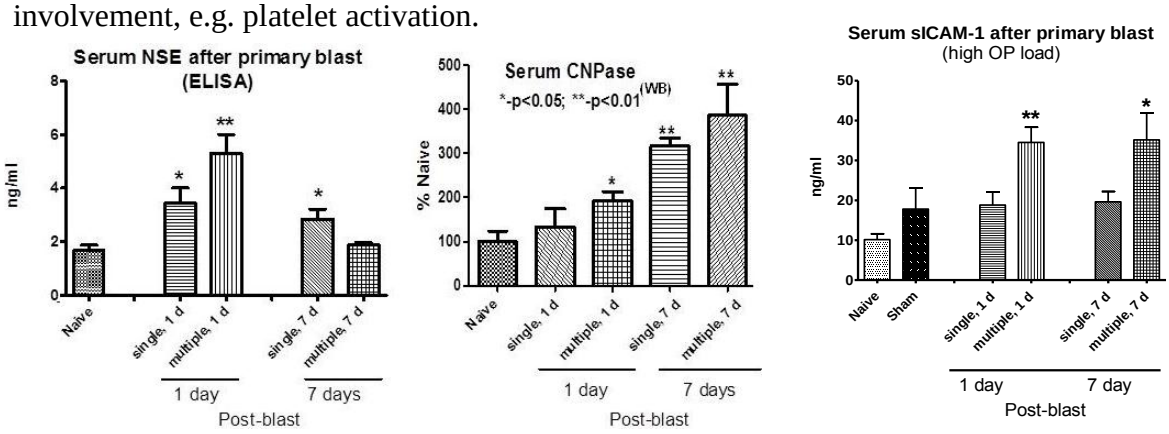

Figure 5 NSE, sICAM and CNpase after single and multiple primary blast

The time-course of NSE, sICAM and CNPase was characterized after single and repeated moderate primary blasts. Biomarker levels rose significantly as a rapid response at day one post-blast, with the CNPase and NSEs after repeated blast exposures elevating further over single

blast. The appearance of characteristic proteins in circulation may reflect deterioration of the BBB and can be used for assessment of injury accumulation. However, triple consecutive blast exposures did not produce a further elevation in biomarkers at 7 days post exposure compared to a single blast. At this time point, their increased levels depend on the cell-specific origin of biomarkers and that stage of injury, rather than reflecting a cumulative blast load.

Task 8 is ~90 % complete. Neuropiline-2 and NGF-beta have been identified and are being investigated as chronic biomarkers after multiple blast reflecting neurorepair. They were reported previously and currently we collect more samples to increase sample size.

According to task 8 of Specific aim 4, we identify thrombin biomarkers as additional critical components of blast brain injury and employed Calibrated Automated Thrombography for assays.
The combined data on thrombin activity at different time-points and blast setups are presented in the Table 2.

Table 2. Indices of Thrombin Activity after Exposure to a Primary/Composite Blast Wave Load.

Primary Blast	CAT parameter	Baseline	6 hr post-blast	1 day post-blast	7 days post-blast
	TG max (nM)	121.0 ± 38.0	513.0 ± 44.0*	212.0 ± 68.0*	255.0 ± 49.0*
	t (Peak) (min)	4.8 ±0.19	8.0 ± 0.24*	7.0 ± 0.12*	5.0 ± 0.11*
	t (Start) (min)	1.1 ± 0.07	1.0 ± 0.08*	1.0 ± 0.09*	1.0 ± 0.07*
	t (Mean) (min)	6.4 ± 0.17	5.4 ± 0.18*	4.5 ± 0.15*	4.0 ± 0.13*
Composite Blast	CAT parameter	Baseline	6 hr post-blast	1 day post-blast	7 days post-blast
	TG max (nM)	120.1 ± 7.2	540.0 ± 26.1*	450.0 ± 23.3*	250.0 ± 11.1*
	t (Peak) (min)	5.0 ±0.14	8.0 ± 0.13*	7.0 ± 0.13*	5.0 ± 0.10
	t (Start) (min)	1.2 ± 0.08	1.0 ± 0.07*	1.0 ± 0.06*	1.0 ± 0.06*
	t (Mean) (min)	6.4 ± 0.12	5.5 ± 0.13*	4.5 ± 0.11*	4.0 ± 0.10*

* P value <0.05 vs. naïve samples.

All indices of TG were remarkably affected in all blast exposed rats compared to naïve animals. However, in 'composite' blast exposed animals, TGmax peaked at 6 hr (~4.5-fold vs. control), sustained at 1 day (~3.8-fold increase), and declined to a 2-fold increase over control levels at day 7 post-blast. In rats subjected to primary blast, TGmax also rose to ~4.2-fold of control values at 6 hours, dropped to ~1.7-fold of control levels at 1 d post-blast, and then exhibited a secondary increase in 2-fold of control values at day 7 post-blast (Fig. 2 A).

Other TG indices did not differ significantly between two types of blast exposure. After either 'composite' or primary blast loads the t -Peak times significantly increased compared to control

values while corresponding t-Mean values decreased at both blast setups. The representative overlapped TG tracings after a primary blast wave load are illustrated in Figure 6 B.

The cumulative analysis of the data suggests strong time-dependent stimulation of overall thrombin production by blast exposure.

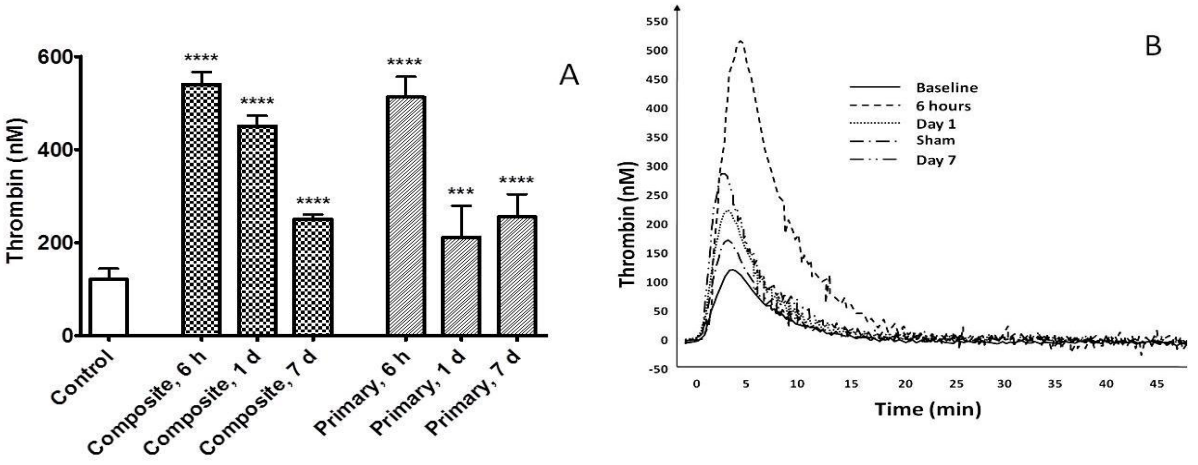

Figure 6 Plasma levels of Thrombin after blast. Concentrations of Thrombin were assessed in rat plasma by CAT technology (A). Representative thrombography tracings after primary blast exposure (B). Data shown are Mean+SEM of at least three independent experiments. *-p<0.001; ****-p<0.0001 vs. control samples**

Concentrations of matrix metalloproteinases MMP-2 (D), MMP-8 (E) and MMP-13 (F) were assessed in rat serum by antibody arrays and ELISA. Please see Materials and Methods for details.

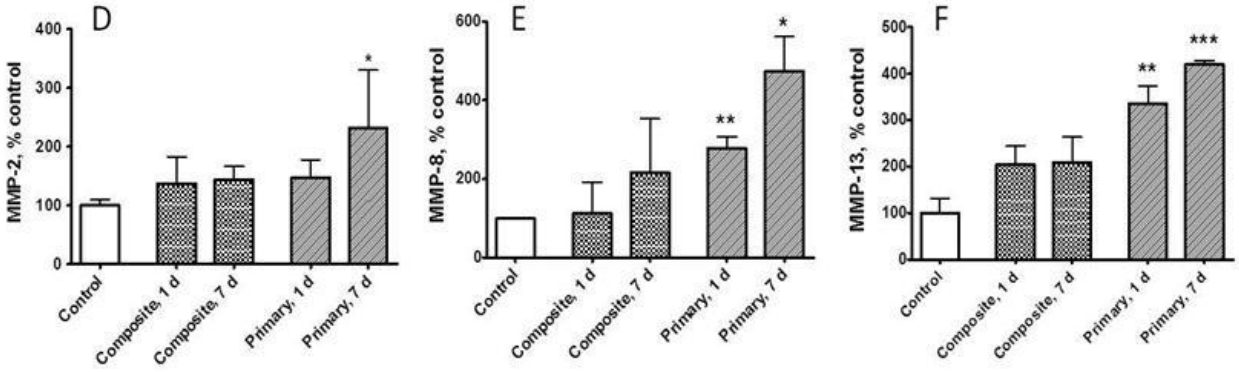

Figure 7 Levels of Matrix metalloproteinases MMP-2 (D), MMP-8 (E) and MMP-13 (F) in serum of rats subjected to moderate primary blast.

Blood was collected from OP-exposed rats at different shock tube set-ups. Data shown are Mean±SEM of at least three independent experiments. *-p<0.05; **-p<0.005; ***-p<0.001 vs. control samples.

In conjunction with vascular/microcirculation biomarkers sICAM and integrin-α/β, this indicates the importance of general inflammatory reactions in response to blast exposure and transition to tissues inflammation.

We assessed Neuroendocrine dysfunctions after single and repeated blast exposure in addition to Orexin A reported previously. Combined data are shown in the table below and detailed in the published paper attached in the appendix:

Table 3. **Catecholamine biosynthetic enzymes and NPY protein in the adrenal medulla of control and overpressure blast injury animals**

Protein	Control (n=4)	OBI (n=4)
TH	100 ± 2%	120 ± 7%*
DβH	100 ± 4%	125 ± 9%*
NPY	100 ± 9%	191 ± 20%*

Values are mean ± SE of 4 rats/group expressed as percentage of control. The level of the averaged control for each protein is arbitrarily set to 100 with SE adjusted proportionally with remaining groups normalized to the level in control. *Significantly increased versus Control; P=0.022 (TH), P=0.031 (DβH), P=0.006 (NPY). TH = tyrosine hydroxylase, DβH = dopamine β-hydroxylase, NPY = neuropeptide Y.

Key Research Accomplishments:

- The final sensor prototype version 2.0 and signal conditioning circuit have been produced and successfully tested. The proposed prototype has shown the response speed to accurately record the peak overpressure of the blast event as compared to the benchmark PCB sensor.

- A comprehensive evaluation has been done to characterize mild through severe blast TBI and differences revealed between moderate 'Primary' vs 'Composite' blast responses in pathogenic pathways and biomarker responses. While both type of exposures were characterized by strong gliosis, 'composite' blast on the head (accompanied by head acceleration) produces significantly higher neuronal injury.

- We have shown that systemic, vascular, neuroinflammatory and neuroendocrine responses are essential components in responses to blast in general and primary OP exposure in particular:

    - Orexin A, sICAM and Neuropilin-2 (NRP-2) have been confirmed as the most prominent biomarker candidates having subacute and chronic diagnostic utility

    - We demonstrate increases in tissue metalloproteinases MMP-2 (D), MMP-8 (E) and MMP-13 (F) after primary blast further supporting systemic responses to blast exposure. This was co-incident with increases in integrin-α/β,

    - Orexin A was dramatically elevated at day 1 and 7 post-blast, no differences in multiple blast responses vs. single were found. In contrast, both sICAM and NRP-2 serum levels after multiple blasts were higher than after single at day 1 and 7 post-blast.

    - Multiple blasts significantly augmented increased levels of GFAP, UCH-L1 and NSE vs single blast at 1 day post blast. No augmentation was found at 7 day post-blast. On the other hand, serum CNPase, sICAM and NRP-2 after multiple blasts were significantly augmented vs. single blast both at 1 day and 7 days post exposure.

    - Multiple blast further augmented the Dye accumulation suggesting that BBB has a tendency and ability to reopen after repeated blast exposures.

- Novel thrombin-generated biomarkers of blast exposure linking microcirculatory, inflammatory and coagulation disorders have been assessed and their importance in pathogenesis has been demonstrated.

- A substantial activation of catecholamine biosynthetic enzymes and Neuropeptide Y has been shown supporting a profound neuroendocrine responses after blast exposures.

Reportable Outcomes for the project period September 16, 2012-September, 2013

1. A previous paper for special topic Blast-induced Neurotrauma "**Neuro-glial and systemic mechanisms of pathological responses in rat models of primary blast overpressure compared to "composite" blast.**" by Svetlov SI, Prima V, Glushakova O, Svetlov A, Kirk DR, Gutierrez H, Serebruany VL, Curley KC, Wang KK, Hayes RL. has been published in Frontiers in Neurol. 2012; 3:15,
   This paper has been already cited by

   Zou et al. in Primary blast injury-induced lesions in the retina of adult rats; J Neuroinflammation. 2013; 10: 79. doi: 10.1186/1742-2094-10-79 and by

   Mac Donald et al. in Cerebellar White Matter Abnormalities following Primary Blast Injury in US Military Personnel PLoS One. 2013; 8(2): Published online 2013 February 7. doi: 10.1371/journal.pone.0055823

2. A paper has been published: Prima V, Serebruany VL, Svetlov A, Hayes RL and Svetlov SI Impact of Moderate Blast Exposures on Thrombin Biomarkers Assessed by Calibrated Automated Thrombography in Rats J Neurotrauma. 2013 Oct 4. [Epub ahead of print] PMID: 23805797 **see Appendices**

3. A paper has been published: Tümer, **Svetlov SI,** Whiddenh M, Kirichenko N, Prima V, Erdos B, Sherman A, Kobeissy F, Yezierski R, Scarpace PJ, Vierck C and Wang K.W. Overpressure blast-wave induced brain injury elevates oxidative stress in the hypothalamus and catecholamine biosynthesis in the rat adrenal medulla. Neuroscience Lett. 2013 Jun 7;544:62-7, **see Appendices**

4. A review has been in press : Kobeissy F, Mondello S, Tumer N, Toklu HZ, Whidden MA, Kirichenko N, Zhang Z, Prima V, Yassin V, Svetlov SI, Wang KKW Assessing Neuro-Systemic & Behavioral Components in the Pathophysiology of Blast-Related Brain Injury. Frontiers in Neurotrauma, 2013 (in press). **see Appendices**

5. Adams S, Condrey JA, Tsai HW, Prima V, **Svetlov SI,** Sumners C, and Davenport PW. **Anxiety Produced in Rats by Over-Pressurization Blast Injury** International Society for the Advancement of Respiratory Pyschophysiology held in Leuven, Belgium, September 22-26, 2012. **see Appendices**

Conclusions

In **the first year** of the project, we generally validated the models of 'composite' blast exposure accompanied by head acceleration vs primary blast load, where peak overpressure 'flows the head through a rostral part of the brain' w/o significant head acceleration. Schlieren optics was used to visualize blast wave interaction with experimental animal. Blast peak overpressure, duration, and impulse on the surface of rats at various orientations to the blast wave were determined and standardized. We began characterizing molecular signatures of blast brain injury and outlines several categories of signatures: neuroglial, microvascular, systemic & neuroinflammation, and neuroendocrine changes.

During the second year, we continued comparing blast load characteristics producing mild through severe TBI of 'composite' with primary blast. Also, we initially characterized brain injury and biomarkers after repeated blast exposure by ELISA, antibody microarrays, and Western blot. Rats were subjected to blast of different magnitude, including primary blast of 50-53 psi kPa overpressure, 75 μsec at the frontal part of the rat's skull. We measured blood accumulation of GFAP and CNPase, neuronal UCH-L1 and NSE, neuroendocrine peptide Orexin A, and Neuropilin-2 at different times post-blast. We demonstrated the importance of the orientation of head/body to blast wave for the response and biomarker accumulation. While both type of exposures were characterized by strong gliosis, 'composite' blast on the head (accompanied by head acceleration) produces significantly higher neuronal injury. We also revealed several systemic and microcirculatory inflammation biomarkers such as L-selectin and s-ICAM involved in molecular mechanisms of blast-induced injury. The FIT prototype sensor (version 1) to capture multiple blast exposures was designed, built and successfully tested. We performed preliminary calibration and evaluation of sensor in rats after multiple blasts as a series of 3 exposures, with a 45 min to 1 hr recovery between each blast. Repeated blasts augmented increased levels of GFAP, UCH-L1 and NSE vs single blast at 1 day post blast. No augmentation was found at 7 day post-blast. On the other hand, serum CNPase after multiple blasts was significantly augmented vs. single blast both at 1 day and 7 days post exposure.

In year 3, we completed a comprehensive evaluation of mild through severe blast TBI and differences revealed between moderate 'Primary' vs 'Composite' blast responses in pathogenic pathways and biomarker responses. While both type of exposures were characterized by strong gliosis, 'composite' blast on the head (accompanied by head acceleration) produces significantly higher neuronal injury. The final version of improved prototype (final version) of cumulative blast sensor and signal conditioning circuit has been produced and tested at Banyan Biomarkers November/December, 2013 and January/February 2013. We were capable of capturing at least 3 consecutive blasts and integrate a cumulative load. Serum CNPase and sICAM after multiple blasts was significantly augmented vs. single blast both at 1 day and 7 days post exposure. In contrast, repeated blasts cumulated the increase of GFAP, UCH-L1, NSE and NRP-2, but not Orexin A at day 1 only, while at 7 days the cumulative effects were much lower.
Novel thrombin-generated biomarkers of blast exposure linking microcirculatory, inflammatory and coagulation disorders have been assessed and their importance in pathogenesis has been demonstrated. A strong correlation has been shown with integrin α/β. A substantial activation of catecholamine biosynthetic enzymes and Neuropeptide Y has been shown supporting a profound neuroendocrine responses after blast.

The status of ongoing tasks and plan for the next period:

Task 4: Assess brain injury characteristics upon exposure to repeated low level blast; determine cumulative blast load-injury correlations. This task has been ~90 % completed. Due to operational and logistical issues, we slightly modified blast exposure regimen. Currently, we expose rats to level 1 blast 3 times with 45-60 min interval. Final experiments and analysis of multiple exposures and mTBI parameters is under way and planned to be accomplished.

Specific Aim 3: Assess and compare blood levels of existing and our novel biomarkers of TBI to identify their diagnostic utility overlap for blast-related mTBI during a single and cumulative exposure.

Task 5: Determine the levels of S100β, Neuron-specific enolase (NSE), MBP, GFAP in plasma/serum at different times during development of mTBI, depending on cumulative blast load upon single and multiple exposures. Compare putative biomarker profile in mTBI through severe blast TBI with mechanical brain injury (CCI). **(Months 6-36)**

Task 6: Examine plasma/serum levels of our novel biomarkers UCH-L1, MAP2, SBDP145 in blood after multiple blast mTBI through severe TBI. **(Months 8-39)**

Analyze putative biomarkers and cumulative blast load metrics measured by detecting/recording device using paired statistical analysis (MANOVA). Incorporate biomarker injury level correlations in blast load injury scale.

Current status: overall, this Aim has been ~90 % completed. We have decided to omit MBP measurement (task 5) and replace it with CNPase better reflecting demyelination disorders after blast exposure.

Task 7: Determine critical molecular components of targeted pathological pathways in response to repeated mild blast.

Current status: this task in the specific aim 4 has been slightly modified. Specifically, eNOS and iNOS, analysis omitted, while NSE assays has been included and performed. The task is ~80 % complete and is going on schedule.

Task 8: Identify additional critical components relevant to blast brain injury and indicative of adaptive/regenerative responses. Current status: NRP-2 and NGF have been investigated as chronic biomarkers after multiple blast reflecting neurorepair. We added thrombin biomarkers measured by Calibrated Automated Thrombography (CAT). This task is ~90 % complete.

Status of deliverables: (1) Panel of biomarkers is projected to be completed on schedule (Month 48 of the project). (2) Prototype of cumulative blast impulse-energy sensing package v2 (CBI-ESP) has been produced, grading for cumulative injury is the main goal for next year.

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APPENDICES

EXPERIMENTAL FRAMEWORK FOR CUMULATIVE BLAST DETECTION AND DATA ACQUISITION FOR ASSESSMENT OF BLAST-RELATED INJURY IN ANIMAL STUDIES

Cumulative Blast and Impulse Exposure Sensor Package (CBI-ESP)

Version 2.0 rev B

USER MANUAL

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Jan 3rd , 2013

1 Operating Instructions

The Cumulative Blast and Impulse Exposure Sensor Package (CBI-ESP) is a miniaturized sensor package designed to measure multiple blast events and record cumulative blast exposure data to an external SD card. The data is stored as text files of pressure versus time. The CBI-CSP data acquisition board can accommodate up to two sensor boards, and is programmed to acquire both channels for a fixed time when a pre-programmed trigger pressure value is reached.

1.1 Handling and setup

Like most electronics, the main pressure board is sensitive to static electricity. Always store the main board inside the supplied static protection bag. The sensor board and DAQ board are connected to each other by a miniature wiring harnesses. These harnesses are very delicate and should be handled with care. Do not pull from the wires when trying to disconnect any pin headers. Instead, please pull *from the edge of the white plastic header connectors*, which have a rim to pull using your fingernails.

The system is composed of the following components:

1. The data acquisition board (DAQ board)
2. The sensor board
3. Power source (Lithium polymer rechargeable battery)

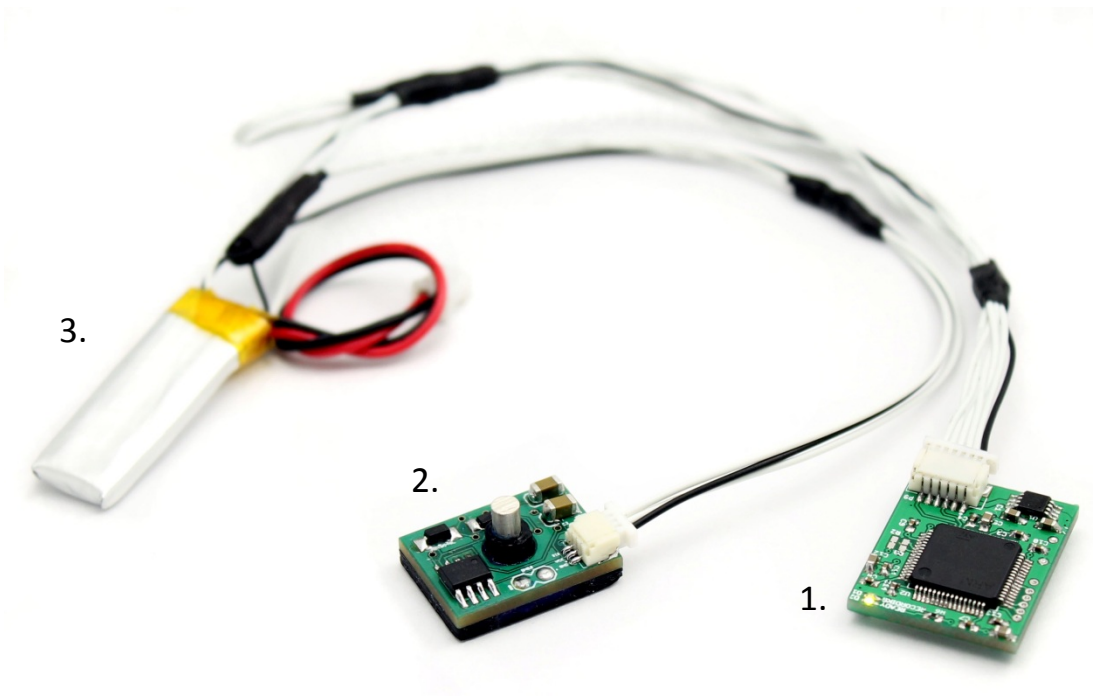


Figure 1: Complete system

1.2 Powering up

1. Insert a micro SD card formatted in FAT16 format, with the required configuration file in it (see Section 2.2.2) into the card holder located in the underside of the DAQ board. Simply push the card in and the socket will click.

2. Connect the DAQ board to the sensor board
3. Wait a few seconds to allow stabilization of the sensor
4. Connect the battery to the DAQ board. After connecting the battery you should see a steady green light in the DAQ board, which indicates that power is adequate and the board is operational, waiting to trigger. If the light is not steady green, refer to 2.2.1. If a pre-trigger occurs, wait until the data is fully written into the SD card and observe its behavior: the RED LED must flash shortly and GREEN LED must turn steady on. If the pre-triggering keeps occurring, check the value of the trigger (Section 2.2.2)

1.3 Data Conversion

Data is stored as raw text by the analog to digital conversion device. Each pressure value is stored as an integer between 0 and 4096. To convert the data to pressure, the bias value V_{bias} has to be known. The conversion to pressure is given by:

$$P = \left(V_{sample} - V_{bias}\right) * \frac{3300}{4096} * \frac{1}{S}$$

Where:

P: measured pressure, in psi

V_{sample}: raw sample (integer 0..4096)

V_{bias}: bias value: integer value corresponding to the atmospheric pressure before the blast event occurs. A good estimate can be obtained from averaging the first 500 microseconds of data (500 samples).

S: Sensitivity of the sensor in mV/psi

2 System Description

2.1 Calibration and System Validation

To verify functionality and accuracy of the CBI-ESP v2 system, a comparison between data from the CBI-ESP v2.0 and a standard off-the-shelf high speed blast pressure sensor (PCB-Piezotronics) was conducted using a National Instruments PXI-8331 high speed data acquisition card for data collection. Both sensors were placed outside the venting cone of the shock tube as shown in Figure 3, (a) and (b). In this configuration, both sensors are expected to read approximately the same pressure event at similar phase, although some minor differences are expected due to reflections from the experimental setup.

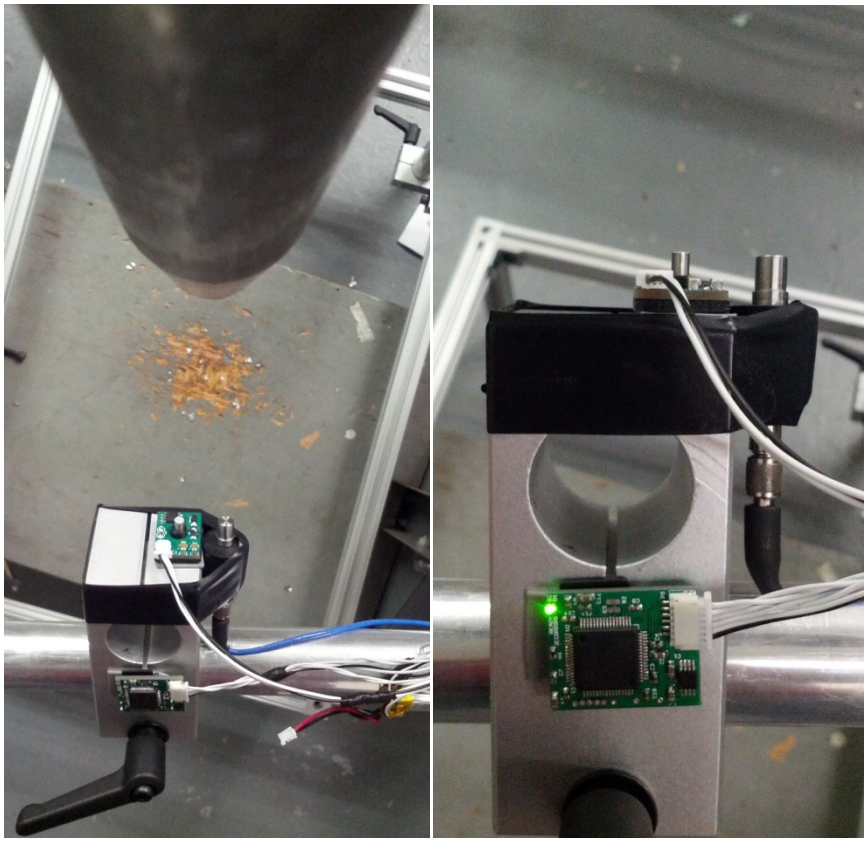
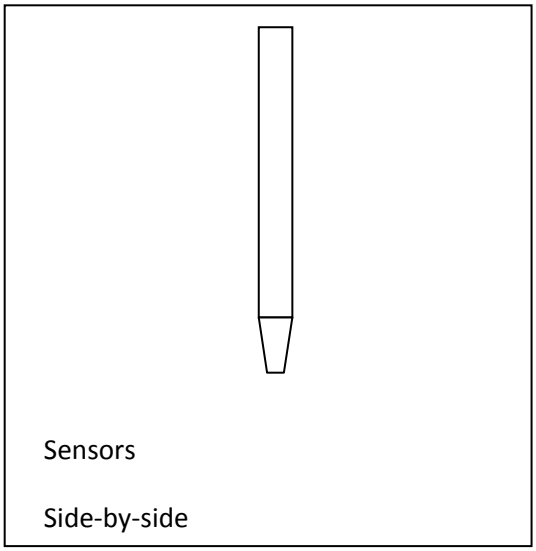


Figure 3 (a): Location of the CBI-ESP sensor board (right) relative to the witness sensor (PCB Piezotronics)

The test was conducted with 1000 psi driver pressure and a driven/driver ratio of 15, using a 0.002 in thick stainless steel shim. The results are shown in Figure 4, showing almost identical peak pressures and very similar pressure traces. None of the signals were filtered. The standard system was sampled using NI-LabView at 10 Megasamples/sec, and the CBI-ESP v2.0 operated at its native sampling rate of 1 Megasamples/sec. The difference between traces is attributed to slight differences in sensor placement.

Figure 3 (b): Location of the pressure sensors relative to the shock tube axis:



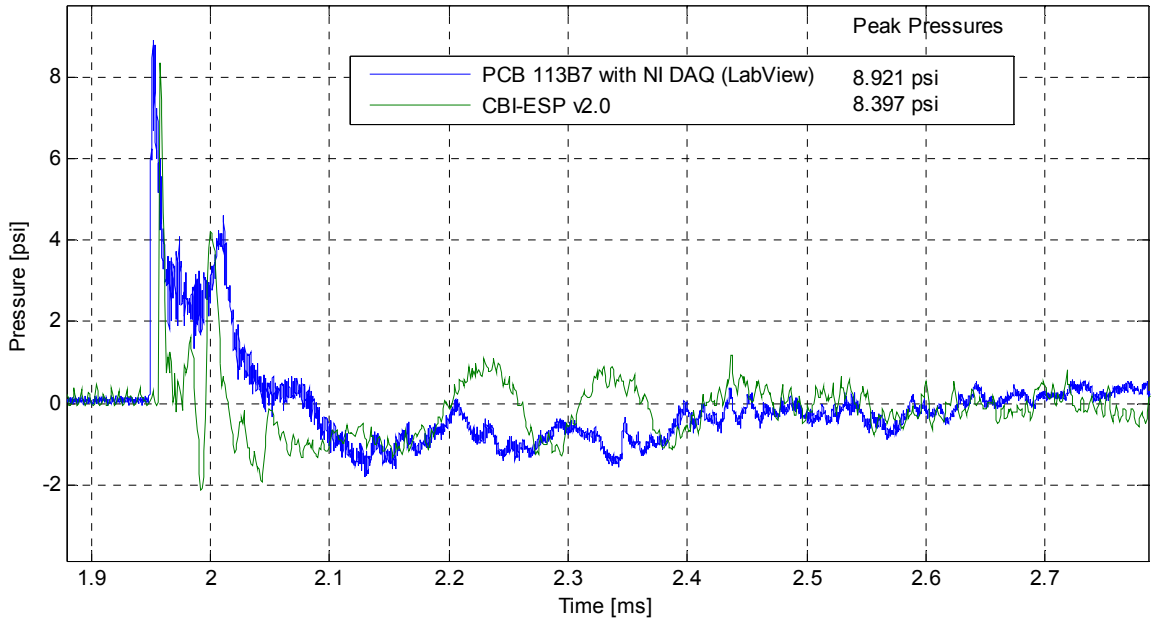


Figure 4: Calibration and validation. Standard blast measurement system (PCB Piezotronics pressure transducer with NI-LabView) compared to the same blast event captured using the CBI-ESP v2.0.

2.2 Data Acquisition Board

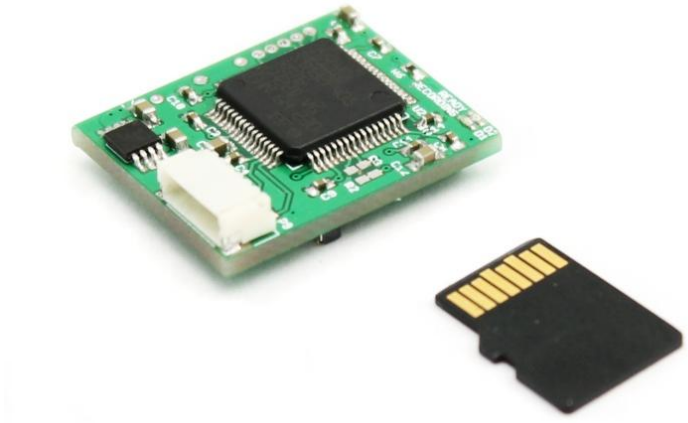


Figure 5: CBI-ESP v2 Data Acquisition Board

The CBI-ESP DAQ board is programmed to collect samples at 1 mega samples/sec from 2 channels simultaneously during up to 20 milliseconds, triggered when either channel goes over a specified programmable trigger value. It stores the data as a text file in a micro SD card, showing two columns: first for channel A, second for channel B. No timestamps are included since the sample rate is fixed. Each data set includes 1000 samples (1 millisecond) of pre-trigger data.

IMPORTANT: When using only one sensor channel, SHORT the UNUSED CHANNEL. DO NOT LEAVE the second channel FLOATING OR IT MAY CAUSE false trigger conditions.

The data acquisition board has a status LED, a 6 pin connector and an SD card slot in its underside.

2.2.1 Status LED

RED LED will flash when:

- No micro-SD card is detected - 250ms period
- Configuration file is missing - 125ms period
- Error creating data file - 50ms period
- After data has been written to the SD card and device is getting ready to collect data again, it will light up once for 250ms.

GREEN LED will be:

- Steady ON, when the board powers up and no error conditions are found. The DAQ board is ready and waiting for trigger.
- Flashing: after a trigger condition occurs, the GREEN LED will flash until it is done writing the pressure event to the micro-SD card, which takes between 30 to 50 seconds depending on the amount of data recorded.

2.2.2 SD Card

The SD card is formatted in the FAT format and needs to have a file called config.txt containing two values separated by a space (4 characters SPACE 2 characters). The first value consists of four digits that represent the trigger level (an integer between 0000 to 4096) that can be changed by the user. When the board detects a value (in either channel) higher than the trigger level indicated in the config.txt file, data acquisition starts.

The second value are two digits that represent the time, in milliseconds, that data collection will last. The possible range is 01 to 20.

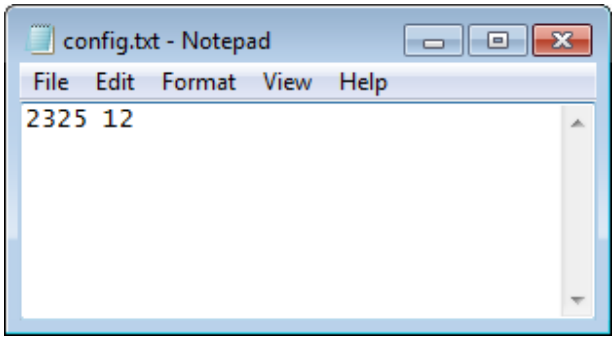


Figure 6: Default values of config.txt file

The collected pressure data will be stored in a separate file called DATA####.txt, where #### is a number between 0000 and 9999. File names for different events are consecutive (DATA0000.txt, DATA0001.txt, DATA0002.txt, and so on). No timestamp can be set to these files since there is no clock present on the board for simplicity.

The data corresponding to the pressure event is stored as text in two columns, space separated, as integers between 0 and 4096 to be converted to psi as described in Section 1.3

The Analog-to-Digital conversion is done in 12-bit resolution, with "0" representing ground, and 4096 representing 3.3V. Thus, the smallest resolution of the DAQ board is 805.7 μV/count.

To convert integer values to voltage, multiply the stored integers by the resolution (3.3 divided by 4096).

2.2.3 Pinout – DAQ Board connector

The pins of the DAQ connector header are shown below:

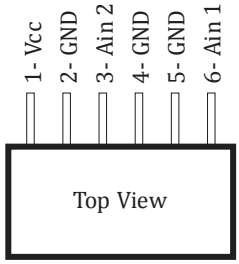


Figure 7: Pin out of the DAQ connector header - Top View

2.3 Sensor Board

Figure 8: Sensor Board

The sensor board boosts the supply voltage (min 2.7V, max 5.5V) to 24V, and limits the current to 4.5mA to feed a Piezotronics 132A31 ICP sensor. It then scales the sensor output voltage to the usable range of the data acquisition board through a gain of 0.2206

The sensor board has the following electrical characteristics:

- Measurement range: zero to 50psi
- Sensitivity: 31 mV/psi ± 1%
- Voltage bias at atmospheric pressure: typically ~1. 8V [1]

For all other electrical characteristics, please refer to the corresponding manufacturer documentation (PCB Piezotronics, 132A31 ICP sensor).

2.3.1 Sensor Board Pinout

The connection pins in the sensor board are as follows:

- Pin 1 GND: Ground
- Pin 2 VCC: Voltage supply (min 2.7V, max 5.5V)
- Pin 3 Vout: Voltage output (0 to 3.3V) [2]

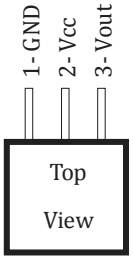


Figure 9: Sensor Board pin out - Top View

[1] Recommended practice: average first 500 pre-trigger samples to obtain accurate voltage bias.
[2] Signal ground is the same as power (battery) ground

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Impact of Moderate Blast Exposures on Thrombin Biomarkers Assessed by Calibrated Automated Thrombography in Rats

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Abstract

Severe blast exposures are frequently complicated with fatal intracranial hemorrhages. However, many more sustain low level blasts without tissue damage detectable by brain imaging. To investigate effects of nonlethal blast on thrombin-related biomarkers, rats were subjected to two different types of head-directed blast: 1) moderate ''composite'' blast with strong head acceleration or 2) moderate primary blast, without head acceleration. Thrombin generation (TG) *ex vivo* after blast was studied by calibrated automated thrombography (CAT). In the same blood samples, we assessed maximal concentration of TG (TG_{max}), start time, peak time, mean time, and concentrations of protein markers for vascular/hemostatic dysfunctions: integrin α/β, soluble endothelial selectin (sE-selectin), soluble intercellular cell adhesion molecule-1 (sICAM-1), and matrix metalloproteinases (MMP)-2, MMP-8, and MMP-13. Blast remarkably affected all TG indices. In animals exposed to ''composite'' blast, TG_{max} peaked at 6 h ($\sim$4.5-fold vs. control), sustained at day 1 ($\sim$3.8-fold increase), and declined to a 2-fold increase over control at day 7 post-blast. After primary blast, TG_{max} also rose to $\sim$4.2-fold of control at 6 h, dropped to $\sim$1.7-fold of control at day 1, and then exhibited a slight secondary increase at 2-fold of control at day 7. Other TG indices did not differ significantly between two types of blast exposure. The changes were also observed in other microvascular/inflammatory/hemostatic biomarkers. Integrin α/β and sICAM-1 levels were elevated after both ''composite'' and primary blast at 6 h, 1 day, and 7 days. sE-selectin exhibited near normal levels after ''composite'' blast, but increased significantly at 7 days after primary blast; MMP-2, MMP-8, and MMP-13 slightly rose after ''composite'' blast and significantly increased ($\sim$2-4-fold) after primary blast. In summary, CAT may have a clinical diagnostic utility in combination with selected set of microvascular/inflammatory biomarkers in patients subjected to low/moderate level blast exposures.

Key words: animal studies; biomarkers; cerebral vascular disease; traumatic brain injury

Introduction

Blast-related traumatic brain injury (TBI) is the most common combat-related injury that ''has emerged as a leading injury among service members'' on the battlefield,[1] while the proportion of civilian casualties caused by explosives has increased as well.[2] TBI can lead to sustained neuro-somatic damage and neurodegeneration,[3] especially when repeated. As the over-pressurization wave propagates through the body, a blast generates primary damage at gas–fluid interfaces,[4] including pulmonary barotraumas, tympanic membrane ruptures with middle ear damage, abdominal hemorrhage and perforation, rupture of the eyeballs, and concussions.[5] Pulmonary barotraumas, together with TBI, are the most common fatal primary blast injuries, including free radical-associated injuries such as thrombosis, lipoxygenation, and disseminated intravascular coagulation. TBI-related coagulopathies substantially increase the risk of death and disability both in civilian[6] and military[7] settings. Current research suggests that blast injury and/or hemorrhage leads to hypotensive and hypoxemic secondary injury and impairs cerebral vascular compensatory responses.[8] Therefore, the effects of mild blast injury on the critical components of hemostasis are of high importance for the development of novel TBI diagnostics and therapeutics, and warrant more in-depth investigation.

Thrombin, or activated factor II, is a protease in the bloodstream that plays a key role in the modulation of hemostasis in general, but specifically in the activation of the coagulation cascade. Thrombin is produced by enzymatic cleavage of prothrombin by activated factor X, and is required to convert soluble protein fibrinogen into insoluble fibrin, promoting formation of a clot.[9,10] In addition,

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thrombin is believed to affect other biological activities in various cell types, including endothelial cells[11] and platelets.[12] Being a potent vasoconstrictor and mitogen, thrombin is recognized as a contributor to both acute and prolonged vasospasm, playing an important role in the pathogenesis of stroke by promoting cerebral ischemia, and/or enhancing risks for intracranial hemorrhage.[13] Several studies identified thrombin as an important contributor to the pathological developments following various injury types.[14,15]

Therefore, assessing thrombin activity represents an attractive, and potentially clinically useful, diagnostic tool for blast-related injury triage.

However, considering fast cleavage and aggressive binding patterns, measurement of thrombin activity is challenging. The most reliable among presently available tests is serial assessment of thrombin in plasma as a function of time, by comparing the fluorescent signal from a thrombin-generating sample using the calibrated automated thrombography (CAT) method, developed by Hemker and colleagues.[16,17] Applying the CAT system, we determined the blast wave-induced effects on multiple indices of thrombin generation (TG) potential and compared them with concomitant changes of several other markers of coagulation/inflammation vessel wall crosstalk. Using these proteins as a supplementary biomarkers panel for TBI diagnostics can validate and support otherwise injury type-nonspecific CAT data.

Methods

Blast generator design and setup

The compressed air-driven shock tube, capable of generating a wide range of controlled blast waves, has been described in detail previously.[18] The tube consists of two sections: high-pressure (driver) and low-pressure (driven) separated by a diaphragm. Peak overpressure (OP), composition, and duration of the generated high pressure shockwaves are determined by the shock tube configuration, including thickness, type of diaphragm material, driver/driven length ratio, and the initial driver pressure at the moment of diaphragm rupture. In the presented series of experiments, we employed different spatial setups as will be described subsequently. The blast pressure data were acquired using PCB Piezoelectric blast pressure transducers and LabView 8.2 software. A National Instruments 1.25 M samples/sec data acquisition card was used to acquire data from multiple channels. The rat head images during the blast event were captured at 40,000 frames/sec using a high speed video camera (Phantom V310, Vision Research, Wayne, NJ).

Animal exposure to a controlled blast wave

Modeling of the primary blast and the ''composite'' OP load was achieved by variable positioning of the target versus the blast generator. All rats were anesthetized with isoflurane inhalations, described previously in detail. After reaching a deep plane of anesthesia, they were placed into a holder exposing only their heads (body-armored setup) at a distance 5 cm below the exit nozzle of the shock tube. Rats were positioned either directly on the shock tube axis ($n=5$) to expose them to the ''composite'' blast including the compressed air jet (Fig.1 A, B) or at the 45 degree angle to it ($n=6$) for exposure only to the primary blast wave (Fig.1 D, E). Animals were then subjected to a single blast with a mean peak OP of 230–380 kPa at the target. The exact static and dynamic overpressure values depending upon the angle and distance of rat head from the nozzle of shock tube were established during the prior calibration tests. The control group of animals ($n=4$) underwent the same treatment (anesthesia, handling, recovery), except they were not exposed to a blast.

Blood collection

At the required time points following blast exposure, animals were euthanized according to guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida. With the animal under isoflurane anesthesia, blood was withdrawn directly from the heart with an 18 gauge needle, and processed to obtain plasma and serum. One half of collected blood aliquot was drawn into 0.5 mL Capiject EDTA (K2) tubes (Terumo, Elkton, MD) at room temperature. The Capiject tube was gently inverted three to five times to ensure complete mixing of the anticoagulant. Platelet poor plasma (PPP) was centrifuged at 6000*g* for 15 min at room temperature, and frozen at -80°C until analysis. Another half of the blood aliquot was drawn into Multivette 600 tubes with clotting activator (Sarstedt, Nümbrecht, Germany) and was allowed to clot at room temperature for 40 min. Serum was separated by centrifugation at 10,000*g* for 5 min and frozen at -80°C until analysis. All samples were labeled with a coded number and analyzed by blinded technicians.

Antibody-based assays

Custom Biotin Label-based (L-series) RatAntibody arrays (Ray Biotech, Norcross, GA) were used to assess relative levels of integrin α/β, soluble endothelial selectin (sE-selectin), and matrix metalloproteinases (MMP)-2, MMP-8 and MMP-13 in rat serum following blast exposure. Commercially available Sandwich ELISA kits for soluble intercellular adhesion molecule-1 (soluble intercellular cell adhesion molecule-1 [sICAM-1]; CUSABIO Biotech) were used according to the manufacturer's instructions.

CAT reagents

Fluobuffer containing 20 mM HEPES and 60 mg/mL bovine serum albumin (Sigma, St. Louis, MO) were prepared *ex tempore* on the day of the experiment. Working buffer consisted of 140 mM NaCl, 20 mM HEPES, and 5 mg/mL human serum albumin. The fluorogenic substrate Z-Gly-Gly-Arg-amino-methyl-coumarin (Bachem, Bubendorf, Switzerland) was solubilized in pure dimethylsulfoxide (DMSO, Sigma, St. Louis, MO). The PPP reagent with a content of 5 pM tissue factor, and the thrombin calibrator (Thrombinoscope BV, Maastricht, Netherlands), was provided by Diagnostica Stago (Parsipanny, NJ).

CAT

Measurement of TG potential was performed using the CAT system. The validation details of the method are described elsewhere.[16,17,19] Briefly, for each experiment, a fresh mixture of fluobuffer and CaCl2 solution was prepared and incubated for 5 min at 37°C. After 5 min, 75 μL of the Fluo-DMSO-solution were added, mixed and incubated for a further 5 min. The resulting clear solution was referred to as FluCa. PPP reagent was solubilized with 2 mL deionized water. Twenty microliters of this trigger solution were put into each sample well of a 96 well round-bottom microtiter plate made of polypropylene (Nunc, Roskilde, Denmark). After reconstitution with 1 mL sterile water, the thrombin calibrator was used in each experiment to compare the simultaneously measured thrombin activity in the sample with that from a known and stable concentration in the calibrator well. Finally, 80 μL of plasma were put into each well. The 96 well plate was then placed in the fluorometer (Fluoroskan Ascent, Thermolabsystems OY, Helsinki, Finland) with an excitation filter at 390 nm and an emission filter at 460 nm. The automated dispensing of 20 μL FluCa indicated the onset of measurement of thrombin indices. Each well was measured every 20 sec for the duration of 40 min. Each experiment was performed fourfold. We used Analysis Software from Diagnostica Stago, Inc. (Parsippany, NJ) to assess four indices, namely TG_{max}

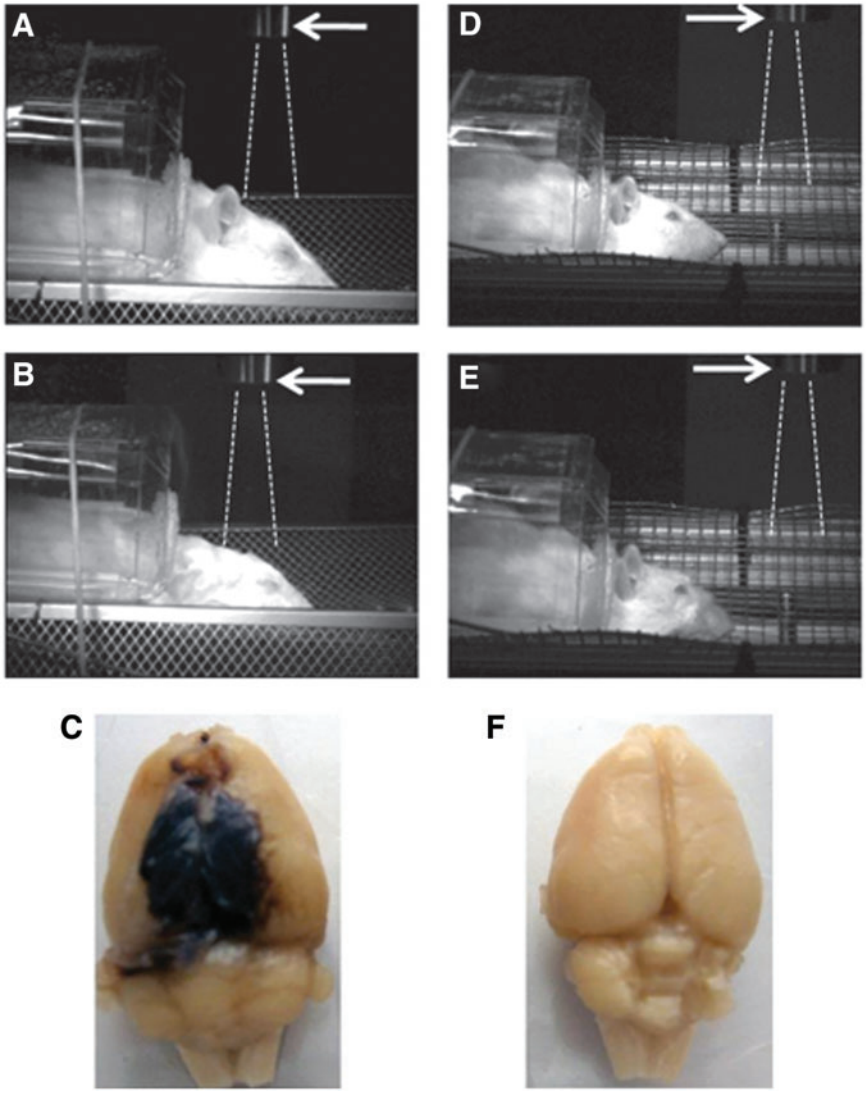

FIG. 1. Rat models of brain injury with ''composite'' or primary blast overpressure. High speed video images recorded before **(A, D)** and after **(B, E)** blast wave passage illustrate rat head movement on ''composite'' on-axis **(A, B)** versus primary off-axis **(D, E)** blast wave load for 10 msec. Arrows indicate the shock tube exit. Dashed lines depict trajectory of compressed air jet. Brain pathomorphology after head-directed exposure to blast wave: anesthetized rats were subjected to a ''composite'' **(C)** or a primary **(F)** blast overpressure load as described in the Methods section. Forty-eight hours after exposures brains were perfused *in situ*, removed, and recorded. Gross pathology: typical focal intracranial hematomas observed following ''composite'' overpressure load of 230–380 kPa. Color images are available online at www.liebertpub.com/neu

(max concentration of TG), start time (t-start) peak time (t-peak), and mean time (t-mean).

Statistical analysis

The Mann–Whitney *U* test was used to analyze nonparametric data. Normally distributed data were expressed as mean ± SD, and skewed data as median (range). All *p* values were two sided, with the significance level set at 0.05. Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA).

Results

Blast-induced gross pathology

The high speed video recordings shown in Figure 1 present different biomechanics of target movement on the load of the ''composite'' or primary blast. Significant head acceleration and deformation after ''composite'' blast exposure (Fig. 1 A, B) were accompanied by typical focal and massive intracranial hematomas and brain swelling. The hemorrhages and hematomas developed within hours after impact and appeared visibly through the undamaged skull at 24–48 h after blast exposure (data not shown). The size of hematomas varied significantly in different rats and formed a capsule at 5 days post-blast, as shown in one of the most damaged rat brains after *in situ* perfusion (Fig. 1 C). The intracranial blood accumulation partially resolved at day 14 in a majority of rats observed (data not shown). On the other hand, primary blast exposure in the described model did not lead to noticeable hematomas.

Thrombin biomarkers

The combined data on TG potential at different time points and blast setups are presented in the Table 1.

Table 1. Indices of Thrombin Activity after Exposure to a Primary/Composite Blast Wave Load

	CAT parameter	*Baseline*	*6 h post-blast*	*1 day post-blast*	*7 days post-blast*
Primary blast	TG_{max} (nM)	121.0 ± 38.0	513.0 ± 44.0*	212.0 ± 68.0*	255.0 ± 49.0*
	t (peak) (min)	4.8 ± 0.19	8.0 ± 0.24*	7.0 ± 0.12*	5.0 ± 0.11*
	t (start) (min)	1.1 ± 0.07	1.0 ± 0.08*	1.0 ± 0.09*	1.0 ± 0.07*
	t (mean) (min)	6.4 ± 0.17	5.4 ± 0.18*	4.5 ± 0.15*	4.0 ± 0.13*
	CAT parameter	*Baseline*	*6 h post-blast*	*1 day post-blast*	*7 days post-blast*
Composite blast	TG_{max} (nM)	120.1 ± 7.2	540.0 ± 26.1*	450.0 ± 23.3*	250.0 ± 11.1*
	t (peak) (min)	5.0 ± 0.14	8.0 ± 0.13*	7.0 ± 0.13*	5.0 ± 0.10
	t (start) (min)	1.2 ± 0.08	1.0 ± 0.07*	1.0 ± 0.06*	1.0 ± 0.06*
	t (mean) (min)	6.4 ± 0.12	5.5 ± 0.13*	4.5 ± 0.11*	4.0 ± 0.10*

*p value < 0.05 versus naïve samples.
CAT, calibrated automated thrombography.

All indices of TG were remarkably affected in all blast-exposed rats compared with naïve animals. However, in ''composite'' blast-exposed animals, TG_{max} peaked at 6 h (~4.5-fold vs. control), sustained at 1 day (~3.8-fold increase), and declined to a 2-fold increase over control levels at day 7 post-blast. In rats subjected to primary blast, TG_{max} also rose to ~4.2-fold of control values at 6 h, dropped to ~1.7-fold of control levels at 1 day post-blast, and then exhibited a secondary increase to 2-fold of control values at day 7 post-blast (Fig. 2A).

Other TG indices did not differ significantly between two types of blast exposure. After either ''composite'' or primary blast loads, the t-peak times significantly increased compared with control values, whereas corresponding t-mean values decreased at both blast setups. The representative overlapped TG tracings after a primary blast wave load are illustrated in Figure 2 B.

The cumulative analysis of the data suggests strong time-dependent stimulation of overall TG potential by blast exposure.

Blast-induced expression induction of hemostasis-related proteins

Integrin α/β levels in serum were raised at both blast setups, indicating that overpressure wave load is triggering microcirculatory disorders whether it produces head hyperacceleration or not (Fig. 3A). After blast, the integrin α/β levels stayed elevated at both assayed time points: 1 day and 7 days. Soluble E-selectin displayed stable serum levels after ''composite'' blast, but increased significantly at 7 days after primary blast (Fig. 3 B). Soluble ICAM-1 levels were elevated in serum at both blast setups from 6 h to 7 days post-blast, most significantly (approximately fourfold of control) at 6 h after ''composite'' and 7 days after primary blast (Fig. 3 C). MMP-2, MMP-8, and MMP-13 displayed similar post-blast responses: slight elevation of relative serum concentrations after ''composite'' blast and significant increase (~2-4-fold) after primary blast (Fig. 3 D–F).

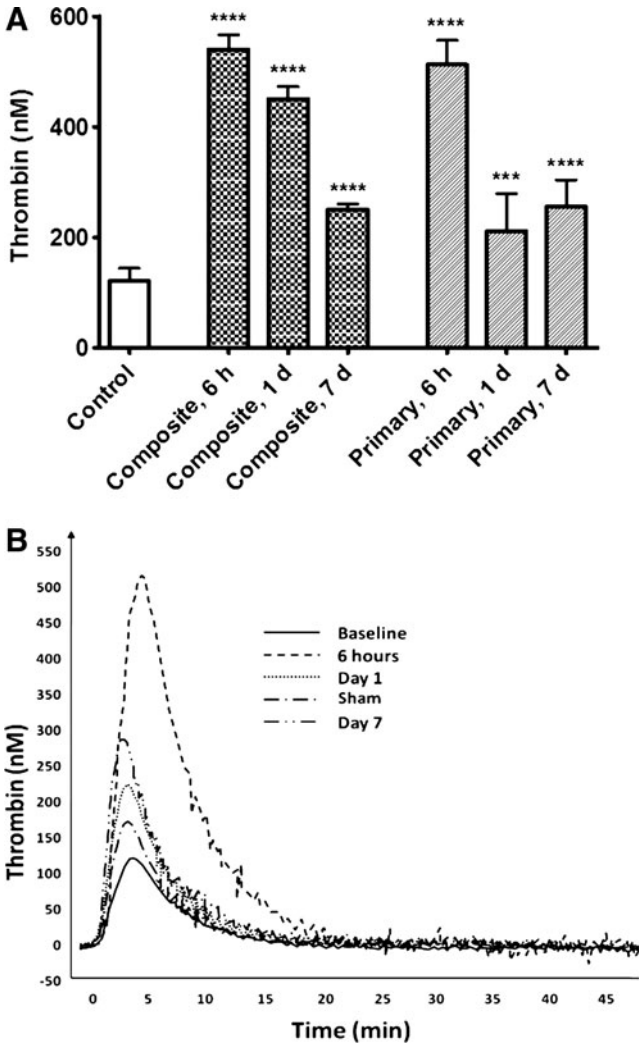

FIG. 2. Plasma levels of thrombin after blast. Thrombin generation potential was assessed in rat plasma by calibrated automated thrombography (CAT) technology **(A)**. Representative thrombography tracings after primary blast exposure **(B)**. Please see Methods section for details. Blood was collected from overpressure (OP)-exposed rats at different time-points and shock tube set-ups. Data shown are mean ± SEM of four independent experiments. ***$p < 0.001$; ****$p < 0.0001$ versus control samples.

Discussion

Our previous studies[18,20] suggested that blast wave composition should be taken into account in the explosive blast modeling with compressed gas-driven shock tubes. Here we explored the impact on hemostasis of two different types of blast: 1) moderate composite (head on-axis) blast with strong head acceleration, and 2) moderate primary off-axis blast load on the frontal part of rat skull without head acceleration. There have been multiple studies[20–23]

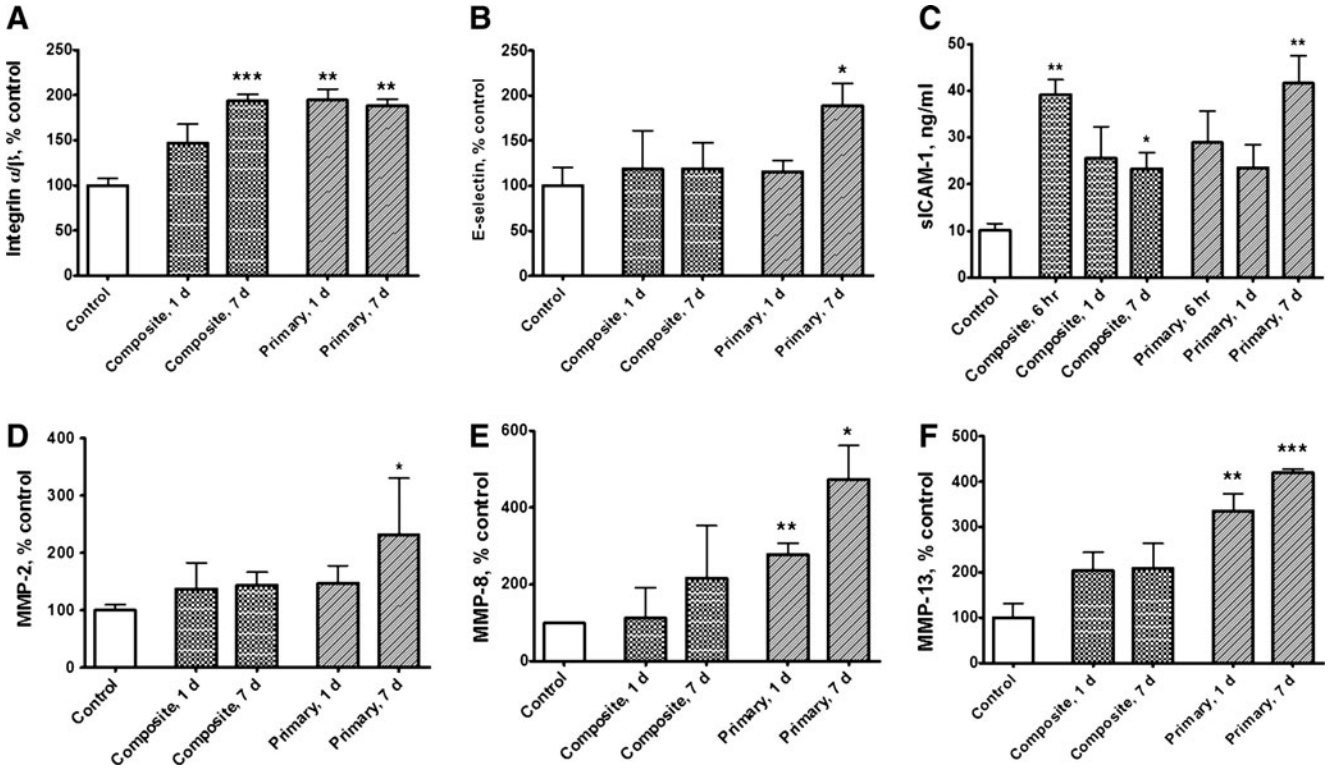

FIG. 3. Serum levels of hemostasis-related proteins after blast. Concentrations of integrin α/β **(A)**, soluble endothelial selectin (E-selectin) **(B)**, soluble intercellular cell adhesion molecule-1 (ICAM-1) **(C)** and matrix metalloproteinases (MMP)-2 **(D)**, MMP-8 **(E)**, and MMP-13 **(F)** were assessed in rat serum by antibody arrays and ELISA. Please see Methods section for details. Blood was collected from overpressure (OP)-exposed rats at different shock tube setups. Data shown are mean ± SEM of four to six independent experiments. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$ vs. control samples.

showing that angular and linear head accelerations (''bobblehead effect'') have much more severe impact than primary blast wave passing through the brain tissue. Moreover, some studies suggest that angular accelerations generate more powerful pressures in the brain than do linear accelerations.[24,25] Also, because of the presence of compressed air jet in the ''composite'' blast wave, the target experienced much higher OP impact resulting in intracranial hematomas (the typical post-blast gross pathology represented in Fig. 1 C).

In mammals, hemostasis is achieved through primary platelet activation-aggregation and secondary coagulation cascade. TBI induces loss of equilibrium in tightly regulated hemostatic systems, which can lead to either hypercoagulable states with microthrombosis and ischemia, or hypocoagulable states with possible progression of hemorrhagic legions.[26] In our studies, only the animal's head was exposed to blast waves, because of a rigid protective shield covering the rest of the body. Nevertheless, hemostasis-related indices were strongly affected in the peripheral blood. TG, the key process in the secondary hemostasis, was strongly affected by blast exposure. Usually thrombin levels are difficult to measure, and TG is commonly assessed indirectly through either enzyme-inhibitor complexes or prothrombin cleavage fragments. In our studies, we employed a novel method, CAT, which has been used recently for analysis of hemostasis in stroke patients.[27] As the data presented in Table 1 and further illustrated by Figure 2 show, all indices of TG were remarkably affected in all blast-exposed rats compared with control animals. An early more than fivefold spike of TG gradually decreased over 7 days post-blast, but still significantly exceeded the control values, suggesting it as a potential candidate for a clinical biomarker.

Following the blast, we observed coincident changes in the other important coagulation and inflammation factors in the hemostasis cascade, which exhibited trends in agreement with TG upregulation. However, contrary to the initial expectations, serum levels of the biomarkers studied after ''composite'' blast with strong head acceleration did not in general exceed corresponding levels after primary blast. In this respect, coagulation/inflammation biomarker data oppose our blast-induced gross pathology findings (Fig. 1A–C), and the existing hypothesis that head acceleration-deceleration resulting from blast forces exerted on the skull (''bobblehead effect'') would be the prevailing cause of persistent brain injury.[23] As shown in Figure 3, serum integrin α/β concentrations were raised after either primary or composite blast exposures and remained significantly elevated up to 7 days post-blast. It is known that the integrins, a large family of cell surface receptors, play pivotal roles in platelet adhesion and aggregation, white cell/endothelium interactions, and platelet-mediated thrombin generation.[28] Our findings are in line with the available data, which indicate that vascular injury is a stimulus for expression of α/β integrins by vascular cells.[29] Concomitant rise of thrombin and integrin α/β reflects important interplay between thrombin and β3-integrins in hemostasis. Thrombin, by binding to G protein coupled, protease-activated receptors, is a potent activator of integrins. Conversely, outside-in signaling through integrins amplifies events initiated by thrombin, and is necessary for full platelet spreading, platelet aggregation, and the formation of a stable platelet thrombus.[30]

As was shown in animal models of TBI, an influx of peripheral blood cells through disrupted blood-brain barrier (BBB) begins within hours after injury.[31] Multiple TBI-related animal studies[32,33] and clinical data[34,35] in agreement with our findings (Fig. 3 B, C) also demonstrate significant elevation in serum of inflammatory cell adhesion molecules, such as sICAM-1 and sE-selectin, which bind to circulating leukocytes and facilitate their migration into the injured brain regions. Endothelial pro-inflammatory processes are potently induced by thrombin.[36–38] Adverse effects of inflammatory response to injury are reflected by highly significant relationship between serum sICAM-1 and poor neurological outcome.[35,39]

Another group of molecules deeply involved in the neuroinflammation processes, MMPs, are known to be rapidly upregulated in patients with TBI,[40] and contribute to BBB breakdown[41] by degrading tight-junction proteins.[42] The consequent increase in blood vessel permeability[43,44] facilitates the development of edema. Our observations that MMP-2, MMP-8, and MMP-13 increase following primary blast wave exposure (Fig. 3 D–F) support the current vision of the diverse mechanisms of MMPs' involvement in brain injury either directly through degradation of brain matrix-substrates or indirectly through interaction with other bioactive molecules,[45] including thrombin[46,47] and integrin.[48] At this point, we do not have sufficient explanation as to why MMPs' levels after primary blast significantly exceed their levels after ''composite'' blast with strong head acceleration. If confirmed by independent studies, this effect may have a special advantage for the detection of mild blast-induced vascular abnormalities in the absence of the ''boblehead effect'' accompanied by severe hematomas.

Only recently has the development of compressed gas-driven blast wave generators with controlled OP enabled quantitative assessment of closed head blast TBI *in vivo*.[18,23,49,50] Analysis of the details of blast wave interaction with the target in the animal models have set it apart both from the civilian accidental TBI cases and from the penetrating brain injuries.[20] Notably, blast-induced closed head injuries are rarely as gruesome as their open counterparts, even though they can be just as damaging. Because these injuries neither puncture the dura mater nor necessarily breach the skull or scalp, they tend to be very hard to detect in the field or even in the hospital, as conventional imaging techniques such as MRI, functional MRI (fMRI), and CT can only detect gross internal deformities. Especially difficult is the objective assessment of mild blast trauma severity when the apparent trauma signs are benign or hidden.[1,3] As discussed previously,[51,52] vasospasm and rapidly developing diffuse cerebral edema leading to intracranial hypertension have been identified among the unique hallmarks of blast-induced closed head injuries encountered in military and civilian settings, which underlines the need for adequate diagnostic tools for hemodynamic and hemostatic abnormalities. Because thrombin, a central molecule in coagulation, is also involved in inflammation,[53] it positions TG among potential biomarkers for predicting neurological outcome after blast-induced TBI. Further human studies would be required to evaluate its clinical applications. Assessing TG potential, in combination with a carefully selected panel of blood biomarkers related to the cerebral hemostasis disruption, may be an attractive and reliable diagnostic tool for mild blast-related injury triage.

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Author Disclosure Statement

No competing financial interests exist.

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Overpressure blast-wave induced brain injury elevates oxidative stress in the hypothalamus and catecholamine biosynthesis in the rat adrenal medulla

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HIGHLIGHTS

- A single OBI was performed in rats to assess the activation of hypothalamic sympatho-adrenal-medullary axis.
- Adrenal medullary catecholamine synthetic enzymes and NPY protein expression as well as plasma NE were elevated.
- NADPH oxidase activity was increased in the hypothalamus.
- TH protein was elevated in the NTS.

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ABSTRACT

Explosive overpressure brain injury (OBI) impacts the lives of both military and civilian population. We hypothesize that a single exposure to OBI results in increased hypothalamic expression of oxidative stress and activation of the sympatho-adrenal medullary axis. Since a key component of blast-induced organ injury is the primary overpressure wave, we assessed selective biochemical markers of autonomic function and oxidative stress in male Sprague Dawley rats subjected to head-directed overpressure insult. Rats were subjected to single head-directed OBI with a 358 kPa peak overpressure at the target. Control rats were exposed to just noise signal being placed at ~2 m distance from the shock tube nozzle. Sympathetic nervous system activation of the adrenal medullae (AM) was evaluated at 6 h following blast injury by assessing the expression of catecholamine biosynthesizing enzymes, tyrosine hydroxylase (TH), dopamine-β hydroxylase (DβH), neuropeptide Y (NPY) along with plasma norepinephrine (NE). TH, DβH and NPY expression increased 20%, 25%, and 91% respectively, following OBI ($P < 0.05$). Plasma NE was also significantly elevated by 23% ($P < 0.05$) following OBI. OBI significantly elevated TH (49%, $P < 0.05$) in the nucleus tractus solitarius (NTS) of the brain stem while AT1 receptor expression and NADPH oxidase activity, a marker of oxidative stress, was elevated in the hypothalamus following OBI. Collectively, the increased levels of TH, DβH and NPY expression in the rat AM, elevated TH in NTS along with increased plasma NE suggest that single OBI exposure results in increased sympathoexcitation. The mechanism may involve the elevated AT1 receptor expression and NADPH oxidase levels in the hypothalamus. Taken together, such effects may be important factors contributing to pathology of brain injury and autonomic dysfunction associated with the clinical profile of patients following OBI.

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1. Introduction

Blast-related traumatic brain injury (TBI) poses a significant concern for military personnel engaged or veterans previously deployed in war zones [3]. The pathophysiology of blast exposure is complex and uniquely different than typical civilian traumatic brain injury as a result of physical trauma or impact to the head. Blast exposure in military situations has various components including: (a) blast overpressure wave-induced injury; (b) secondary injury caused by debris fragments; (c) tertiary injury due to the acceleration or deceleration of the body or body parts due to blast wind or surrounding object; (d) toxic gas, flash burns or intense heat induced bodily injury; and (e) blast noise [3]. Because blast overpressure wave is a primary component of blast-induced organ injury, we previously described an overpressure brain injury (OBI) procedure in rodents using a shock-tube device that can be used as a model for the blast overpressure wave experienced by military personnel [26]. The major effects of OBI have been generally attributed to its external physical impact on the organs, causing internal mechanical damage. The resulting pathophysiological effects include elevated heart rate, blood pressure, respiratory rate, and body temperature [10], as well as cognitive impairment and post-traumatic stress disorder related traits [28].

One recognized pathophysiological consequence of blunt-force-mediated TBI is disruption of autonomic function, resulting in augmented sympathoactivation, but the precise nature of this disruption is not completely understood. Sympathoactivation contributes to systemic stress and cardiovascular complications [3,10]. It is known that TBI is associated with activation of the hypothalamic-pituitary-adrenal (HPA) axis [9]. Another critical participant in the stress response is the hypothalamic sympatho-adrenal-medullary axis [17]. Whether TBI also activates this axis is unknown. Blast induced TBI increases reactive oxygen species (ROS), such as superoxide radicals and nitric oxide [6,29]. In addition, we previously demonstrated that AT1 receptor expression and NADPH oxidase activity in hypothalamus contribute to the activation of the hypothalamic mediated sympathetic outflow [7,8]. Collectively, these data suggest that OBI may stimulate hypothalamic AT1 receptors and NADPH oxidase leading to increased ROS with subsequent activation of the sympatho-adrenal-medullary system.

The nucleus tractus solitarius (NTS) is another brain nucleus that participates in the stimulation of sympatho-adrenal-medullary system following stress [13,17]. The NTS serves as the primary autonomic center that receives viscerosensory inputs from the spinal cord, and cranial nerves project to the NTS through the sensory trigeminal tract. Noradrenergic neurons within the A2 cell group of the NTS, in turn project to the hypothalamus [17].

The sympatho-adrenal-medullary axis leads to marked activation of the AM and sympathetic ganglia characterized by elevated activity of the catecholamine biosynthesizing enzymes such as TH and DβH, resulting in a rise in circulating epinephrine and NE [23]. TH is the rate-limiting step in catecholamine biosynthesis as it catalyzes the hydroxylation of tyrosine to dopamine [20], while DβH catalyzes the conversion of dopamine to NE. In addition to catecholamines, neuropeptide Y (NPY) is synthesized in the AM and is co-released with epinephrine and NE [12,27]. The aforementioned factors, TH, DβH, and NPY are considered the biomarkers of sympathetic nervous system (SNS) activity.

The present study tests the hypothesis that a single exposure to OBI results in increased hypothalamic expression of oxidative stress and activation of the sympatho-adrenal medullary axis. To this end, we measured NADPH oxidase activity and AT1 mRNA expression in the hypothalamus, TH protein expression in the NTS, TH, DβH, and NPY protein expression in the AM as well as plasma NE following a mild-moderate blast overpressure wave.

2. Materials and methods

2.1. Animals

Three month old (250–300 g) male Sprague-Dawley (Harlan Laboratories, Indianapolis, IN) rats were randomly assigned to one of two experimental groups: (1) control ($n = 4$) and (2) brain injury (TBI) induced by blast overpressure wave ($n = 4$). Animals were maintained on a 12:12 h light–dark cycle and provided food and water ad libitum for 2 weeks prior to the experimental protocol. Experiments were conducted according to the Guiding Principles in the Care and Use of Laboratory Animals, and procedures were approved by the local Institutional Animal Care and Use Committee.

2.2. Experimental protocol

Animals in the TBI group were exposed to a single head-directed overpressure blast injury (OBI). A compressed air-driven shock tube was used to expose the TBI rats to a supra-atmospheric wave of air pressure [26]. The tube was separated into two sections: high-pressure (driver) and low-pressure (driven) separated by a metal diaphragm. In these experiments 0.05 mm thick stainless steel diaphragms were used to generate the high pressure shockwaves. The diaphragms were scored with two diagonal and perpendicular lines thus the diaphragms will break away along the score lines without any shrapnel generated. The ratio of driver versus driven section lengths was equal to 15. The driver section was initialized to a pressure of 5170 kPa and maintained at ambient conditions. The diaphragm rupture was initiated by an internal cutter that led to the sudden exposure of a low pressure gas to a gas at a significantly higher pressure resulting in the formation of a shock wave. The blast pressure data were acquired using PCB piezoelectric blast pressure transducers and LabView 8.2 software. A National Instruments 500,000 samples/s data acquisition card was used to acquire data from multiple channels.

Under isoflurane anesthesia rats were placed into a dense Polyethylene holder exposing only their head (body-armored setup) from the exit nozzle of the shock tube with the head positioned directly under the exit nozzle at distance of 5 cm, as described previously [26]. Rats were subjected to a blast wave with a mean peak overpressure of 358 kPa, and a positive pressure phase duration of approximately 10 ms. The noise control animals were positioned 2 m from the exit nozzle thus preventing the rats from experiencing the pressure wave during the diaphragm rupture. The noise duration is about 10 ms with a peak noise level at 100–105 dB. In addition, a control group of naïve animals were included for some western blot analyses.

During the recovery period, the rats are awake and able to walk normally without vocalization of pain. Based on our observation and previous study the above overpressure exposure produces a mild to moderate brain injury. Rats show significant and punctate neurodegeneration as evidenced by increased silver staining in subcortical regions, hippocampus and subthalamic nuclei [26]. Using neurodegeneration cupric silver staining, we observed significant diffused neuronal injury in caudal diencephalon as well as subthalamicus [25].

2.3. Tissue preparation

Six hours following exposure, animals were over-anesthetized with pentobarbital (120 mg/kg ip) and the AM, hypothalamus, and

NTS rapidly removed, immediately frozen in liquid nitrogen and stored at −80 °C until subsequent analyses. Prior to homogenization, AM were decapsulated and the medullae were separated from the cortex.

2.4. NADPH oxidase activity

NADPH oxidase activity was measured with a lucigenin-enhanced chemiluminescence assay using hypothalamus homogenates. Tissue samples were incubated at 37 °C in a spectrophotometer. Relative light units were obtained for 30 min in the presence of NADH (500 μm) and lucigenin (230 μM), and background-corrected values were normalized to protein content determined by DC Bradford.

2.5. Reverse transcriptase-PCR

AT1 mRNA expression was identified in the hypothalamus by using relative quantitative reverse transcriptase-PCR through the use of Quantum RNA 18s Internal Standards kit (Ambion, Austin, TX). PCR was performed by multiplexing AT_1 primers (sense, 5′-CAGCTTGGTGGTGATTGTC; antisense, 5′-GCCATCGGTATTCCATAGC) and 18S primers. The optimum ratio of 18S primer to competimer was 1:9. PCR was performed at 94 °C denaturation for 120 s, 55 °C annealing temperature for 60 s and 72 °C elongation temperature for 120 s for 27 cycles.

2.6. Western blot analysis

AM or NTS were homogenized and assayed for protein levels of the catecholamine biosynthetic enzymes, TH and DβH, along with NPY as previously described [31] using antibodies directed to TH (Pel Freez Biologicals, Rogers, AR), DβH (Novus Biologicals, Littleton, CO), and NPY (Santa Cruz Biotechnology, Santa Cruz, CA). An equal amount of protein for each AM homogenate (1.5 μg protein for TH, 10 μg for DβH and 35 μg for NPY) or NTS homogenates (4 μg protein for TH) was applied to the gels.

2.7. ELISA measurements of norepinephrine

Blood samples were taken via cardiac puncture at the time of sacrifice and centrifuged at room temperature in serum-separating tubes containing 1 mM EDTA and 4 mM sodium metabisulfite. The samples were stored at −80 °C until further analysis by an enzyme-linked immunosorbent assay (ELISA) kit (Rocky Mountain Diagnostics, Inc., Colorado Springs, CO), following the instructions of the manufacturer.

2.8. Statistics

Data are presented as means ± SE. Comparisons between groups for each dependent variable were made by Student's *t*-tests (two-tailed). Significance was established at $P < 0.05$.

3. Results

3.1. Blast injury elevates oxidative stress in the hypothalamus

Changes in the level of oxidative stress in the hypothalamus 6 h following OBI was analyzed by evaluating levels of NADPH oxidase activity. NADPH oxidase activity was significantly increased by 36% following blast injury ($P < 0.05$) (Fig. 1, Top). One known activator of NADPH oxidase activity is the renin–angiotensin II system, thus, we also examined AT1 receptor expression in the hypothalamus. AT1 mRNA expression was significantly elevated following OBI ($P < 0.05$) (Fig. 1, Bottom).

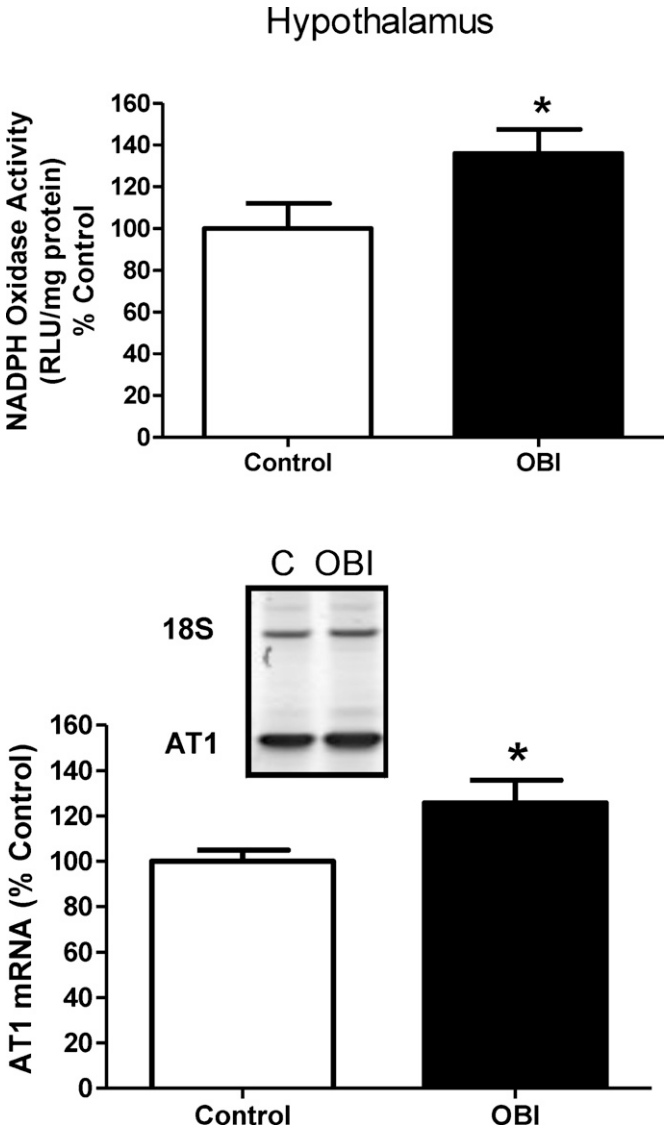

Fig. 1. Effect of OBI on hypothalamic NADPH oxidase activity expressed as relative light units (RLU)/mg protein (Top) and hypothalamic AT1 receptor mRNA expression (Bottom). Values expressed as percentage of control and represent mean ± SE of 4 rats/group. *$P = 0.043$ (Top) and $P = 0.031$ (Bottom) versus corresponding control.

3.2. Blast injury increases tyrosine hydroxylase expression in the nucleus tractus solitarius

We examined TH protein expression following OBI in the NTS. The NTS of the brain stem is one of the most important brain regions involved in neurochemical modulation of stress, cardiovascular control and of central autonomic pathways [5]. Following OBI there was a significant elevation in TH protein expression by 49% compared with control ($P < 0.05$) (Fig. 2, Top).

3.3. Blast injury increases plasma norepinephrine

To determine if the increase in biosynthetic enzyme levels translated to elevated plasma NE levels, plasma NE was assessed following blast injury. Plasma NE was increased by 23% at 6 h post blast injury (Fig. 2, Bottom).

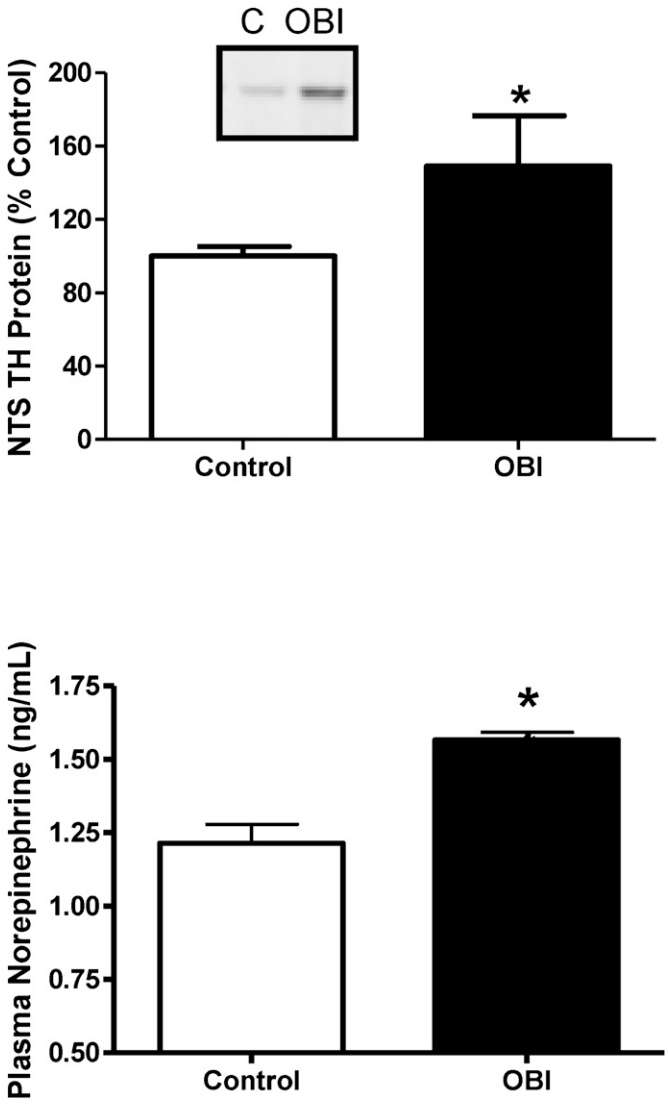

Fig. 2. Effect of OBI on NTS protein level of TH expressed as percentage of control (Top) and plasma norepinephrine (Bottom). Values represent mean ± SE of 4 rats/group. *$P = 0.048$ (top) and $P = 0.002$ (bottom) versus corresponding control.

3.4. Blast injury increases catecholamine biosynthetic enzymes and NPY expression in the adrenal medullae

Sympathetic nervous system activation of the AM was evaluated after blast injury by assessing the expression of catecholamine biosynthesizing enzymes, TH and DβH, along with NPY. Following OBI, TH and DβH expression increased by 20% and 25%, respectively ($P < 0.05$) (Table 1). NPY is synthesized and co-released with catecholamines in the AM and NPY expression correlates with catecholamine biosynthesis. Interestingly, NPY expression was significantly elevated by 91% ($P < 0.01$) following OBI compared with age-matched noise controls (Table 1). We also assessed TH, DβH and NPY expression in the AM of naïve animals; there was no significant difference between the noise control and naïve rats (data not shown).

Table 1
Catecholamine biosynthetic enzymes and NPY protein in the adrenal medulla of control and overpressure blast injury animals.

Protein	Control ($n = 4$)	OBI ($n = 4$)
TH	100 ± 2%	120 ± 7%*
DβH	100 ± 4%	125 ± 9%*
NPY	100 ± 9%	191 ± 20%*

Values are mean ± SE of 4 rats/group expressed as percentage of control. The level of the averaged control for each protein is arbitrarily set to 100 with SE adjusted proportionally with remaining groups normalized to the level in control. TH, tyrosine hydroxylase; DβH, dopamine β-hydroxylase; NPY, neuropeptide Y.

* Significantly increased versus Control; $P = 0.022$ (TH), $P = 0.031$ (DβH), $P = 0.006$ (NPY).

4. Discussion

Overpressure wave exposure with 358 kPa peak pressure used in the present study "on-axis" exposure induces head acceleration and mild to moderate brain injury. The results provide evidence that OBI increases NADPH oxidase activity and AT1 mRNA in the hypothalamus with a parallel increase in TH protein levels in the NTS of the brain stem just 6 h post injury. Moreover, the primary finding is that OBI is associated with increased levels of TH, DβH and NPY protein expression in the AM along with elevated plasma NE level in adult Sprague-Dawley rats.

These data suggest that OBI activates the sympatho-adrenal-medullary axis resulting in elevated adrenal medullary catecholamine synthetic enzymes and NPY protein expression as well as elevated plasma NE. Moreover, the magnitude of the increase in catecholamine biosynthetic enzymes in the present study (20–25%) is similar to that observed in AM and for urinary catecholamines in rats during adaptation to repeated immobilization stress [16], indicating that OBI is a potent stress inducer. It is well known that TH protein increases with the enhanced synthesis and release of catecholamines [30]. Moreover, our demonstration of elevated plasma NE confirms sympathetic activation. In addition, NPY protein level is one biomarker of SNS activity, and there is evidence of a role for NPY in the autocrine regulation of TH expression. NPY expression often increases concomitantly with TH expression. For example, we have demonstrated that carbachol, a mixed nicotinic-muscarinic agonist, stimulates both TH and NPY mRNA expression [27]. NPY may be a part of the homeostatic mechanisms that are involved in the adaptation to stress [11]. Collectively, the present data strongly suggest that post blast injury results in sympathoexcitation.

The effects of brain injury on central and peripheral alterations in the catecholaminergic system have been reported. Within 2 weeks of brain injury, TH protein levels and activity as well as dopamine and NE levels were increased in rat cortical tissue. The increase was transient, and by day 28, TH levels normalized [14]. Others reported a slightly different time course, with cortical TH levels increased at 28 days following post-injury, but with no increase prior to this time point [32], and an acute decrease in dopamine concentrations in the injured cortex at 1 h post-injury, which persisted for up to 2 weeks [19]. Striatal concentrations of dopamine were increased only at 6 h following injury. Hypothalamic concentrations of dopamine and NE increased significantly beginning at 1 h post-injury and persisted up to 24 h for dopamine and 1 week for NE. These investigators speculated that acute alterations occur in regional concentrations of brain catecholamines following brain trauma, which may persist for prolonged periods after brain injury. In humans, elevated plasma NE was reported during the first 3 days of trauma [4]. In another study, elevated plasma NE was detected by day 14, and increased CSF NE levels were decreased at day 6 post-injury [18]. Clearly, NE availability depends on the severity and time after TBI. Adrenoreceptors are also modulated following brain injury. For example, α1a adrenoreceptor mRNA, but not α1b or α1d adrenoreceptor mRNA, is increased in frontal cortex at 2 weeks after TBI [24]. These results indicate that there are markedly conflicting findings as to the functional effects that alter NE availability and influence post-injury cerebral function and cell loss.

We have previously shown that elevated hypothalamic NADPH activity increases blood pressure, indicative of elevated sympathetic activity [7]. Thus, increases in oxidative stress in the CNS may be one mechanism by which blast injury leads to sympathetic hyperactivity and hypertension. NADPH oxidase catalyzes the one electron reduction of oxygen into superoxide using either NADPH or NADH as the electron donor. In the present study, NADPH oxidase activity is significantly elevated just 6 h post OBI. This is similar to a report by Ansari et al. [2], who found significant time-dependent changes in antioxidants as early as 3 h post trauma and paralleled increases in oxidants in hippocampus. In addition, Readnower et al. [21] also reported that 3 h following blast injury 4-hydroxynoneal (4-HNE) and 3-tyrosine (3-NT) were significantly elevated in the hippocampus indicating an increase in oxidative stress.

The renin–angiotensin system (RAS), specifically angiotensin II (ANG II) has also been recognized as participating in various stress-induced responses, including an increase in sympathetic activity and stress-related cardiovascular disorders [22]. Thus ANG II and its receptors contribute to the development of various sympathetic and neuroendocrine responses during stress exposure. In the present report, AT1 expression was significantly elevated following OBI, suggesting the renin angiotensin II system may be involved in the blast injury mediated sympathoactivation. Because stimulation of renin angiotensin II system increases oxidative stress via AT1 receptor stimulation [15], this may provide the mechanism that links OBI and the increase in oxidative stress as well as the downstream sympathoexcitation and cardiovascular dysfunction.

Furthermore, TH protein expression was found to be elevated in the NTS of the brain stem. The NTS is involved in the central autonomic pathway [1]. The exact consequences of elevated TH in the NTS on sympathoexcitation in our experiments are unclear. Neuronal cell bodies which synthesize noradrenaline and adrenaline are found in cell groups within the NTS, including the A2 noradrenergic and C2 adrenergic neurons [5]. These neurons project to the hypothalamus [17] and participate in the modulation of cardiovascular, neuroendocrine, behavioral and metabolic responses to stress [5]. The elevated TH levels in the NTS in the present study may play a role in the sympathetic hyperactivity that is observed following OBI. The elevated oxidative stress in the hypothalamus along with the increased TH protein in the NTS either sequentially or collectively may partially mediate the sympathoexcitation leading to the augmented catecholamine biosynthetic enzymes in the AM.

In summary, our data demonstrate increased TH, DβH and NPY protein expression in the AM as well as elevated plasma NE suggesting that OBI results in increased sympathoexcitation. The mechanism may involve central activation of the sympathoadrenal-medullary axis through increases in AT1 receptors and NADPH oxidase levels in the hypothalamus and elevated TH protein in the NTS. In addition, this data provides evidence for elevated central oxidative stress following OBI. Such effects may contribute to OBI induced autonomic dysfunction. Further studies are needed to explore the detailed mechanisms to determine the effect of overpressure blast-wave on sympathoactivation and to enhance the understanding the pathophysiology of autonomic dysfunction.

Disclosures

None.

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Assessing Neuro-Systemic & Behavioral Components in the Pathophysiology of Blast-Related Brain Injury

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Assessing Neuro-Systemic & Behavioral Components in the Pathophysiology of Blast-Related Brain Injury

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Running Title: Pathophysiology and Biomarkers of Blast-Related Brain Injury

Abstract

Among the U.S. military personnel, blast overpressure (BOP) injury is among the leading causes of brain injury. During the past decade, it has become apparent that even blast injury as a form of mild traumatic brain injury (mTBI) may lead to multiple different adverse outcomes, such as neuropsychiatric symptoms and long-term cognitive disability. A major feature of mild blast TBI is its complex manifestation occurring in concert at different organ levels involving systemic, cerebral, neuronal and neuropsychiatric responses; some of which are shared with other forms of brain trauma such as acute brain injury and other neuropsychiatric disorders such as PTSD. The pathophysiology of blast injury exposure involves complex cascades of chronic psychological stress, autonomic dysfunction, neuro/systemic inflammation which renders blast injury as an arduous challenge in terms of diagnosis and treatment as well as identification of sensitive and specific biomarkers distinguishing mTBI from other non-TBI pathologies and from neuropsychiatric disorders with similar symptoms. This is also due to the "distinct" *but shared* and partially identified biochemical pathways and neuro-histopathological changes that might be linked to behavioral deficits. Taken together, due to the complexity of the various pathological mechanisms involved in blast injury, this article aims to provide an overview of the current status of the cellular and pathological mechanisms involved in blast overpressure injury and argues for the urgent need to identify potential biomarkers that can hint at the different mechanisms involved.

Introduction

Traumatic Brain Injury (TBI) represents a major public health problem with an over 150,000 military personnel diagnosed with mild form of TBI (mTBI), due to the exposure to blast resulting in a wide range of neurological and psychological symptoms (Hoge et al., 2008;Ling et al., 2009). Blast-related BIs (bTBI) can be provocatively described as "a silent epidemic of an invisible wound". Current Explosive mechanisms (improvised explosive devices [IEDs], landmines, rocket-propelled grenades [RPGs]) are believed to account for 56–78% of Operation Enduring Freedom (OEF), Operation Iraqi Freedom (OEF) and Operation New Dawn (OND) related injuries (Owens et al., 2008;Sayer et al., 2008). This has led to labeling the blast-induced TBI (bTBI) as the signature brain injury for combat troops in today's military (Okie, 2006;Bhattacharjee, 2008).

Between 2000 and 2010, the Department of Defense (DoD) reported approximately 200,000 head injuries as a consequence of combat-related incidents as well as events occurred in a non-deployed environment (civilian injuries) (DePalma et al., 2011). However, even this number may be an underestimate due to the fact that the majority of blast-related mild TBIs go misdiagnosed and untreated as a consequence of inappropriate approaches of screening, no validated diagnostic criteria or specific detectable abnormalities and clinical symptoms, and lack of diagnostic tools. Acute blunt penetrating injuries comprised 2.8% of this total, the rest were classified as mild TBI (DePalma et al., 2011).

Out of more than 8,000 cases of traumatic brain injury (TBI) reviewed by the Defense and Veterans Brain Injury Center, ~ 50% were related to blast-related barotrauma (Benzinger et al., 2009). The clinical features observed in mTBI resulting from blast exposure vary, these include: headache, fatigue, tinnitus and irritability which have been highly recognized in recent conflicts. Blast overpressure injury has been considered the main cause of both morbidity and mortality in neurotrauma (Shanker, 2007;Long et al., 2009). Furthermore, blast TBI has been the center for military medical concern in the context of polytrauma, since blast-induced injury, due to its complex components (*primary, secondary, tertiary* and *quaternary* injuries) is often accompanied by hemorrhagic blood loss, multiple fractures, burns and systemic injury as well as traumatic brain injury (DePalma et al., 2005;Belanger et al., 2011;Schultz et al., 2011).

The recognition of the high incidence and impact of bTBI; in addition, to the need for a more accurate diagnosis and effective therapeutic interventions, led to an impressive number of experimental and human blast injury studies aiming at investigating the complex interconnected pathways involved in the blast-induced neuropathological/behavioral changes.

This review will focus on three major questions: (i) What is the experimental and human evidence that blast is associated with progressive alterations in the brain and via what mechanism(s)? (ii) What is the relation between blast-induced brain injury and the development of neuropsychological disorders such as post-traumatic stress disorder (PTSD)? (iii) What are the biochemical markers that can identify, track and predict the injury and symptoms observed in patients exposed to blast injury?

Biomechanics of Blast Injury

Blast overpressure (BOP)-induced injury results from an explosion characterized by an abrupt release of energy in such a short period of time within a small volume creating a non-linear shock and pressure wave (Moore and Jaffee, 2010). The blast shock wave of the primary blast is solitary supersonic pressure wave (peak overpressure) characterized with a rapid (sub-milliseconds/msec) increase in pressure followed by sharp fall in pressure, often to sub-atmospheric levels before returning to ambient pressure (Brode, 1959;Elsayed, 1997). This is coupled with the 'blast wind' (forced super-heated air flow) that gives rise to a very large volume of gas that may throw victim's body against other objects. Blast wind, along with the shock wave are the main components of the 'blast wave" (Kirkman et al., 2011;Lemonick, 2011).

Blast can cause four different types of insults: (i) the *primary injury* resulting from the BOP waves due to the shock-wave overpressure and under pressure. This event is usually associated with contusion, edema, hemorrhage, and diffuse axonal injury (DePalma et al., 2005;Warden et al., 2009;Arun et al., 2011;Kirkman et al., 2011). (ii) The *secondary injury* that is due to shrapnel or hard objects propelled. (iii) The *tertiary insult* involves head movement coupled with acceleration/deceleration as a result of blast wind and finally (iv) the *quaternary insult* resulting from thermal burns or the probable use of toxic gases or chemicals. Compared to previous past conflicts, the majority of war zone wounds have been attributed to secondary blast injury (shrapnel propelled by explosions), while tertiary and quaternary blast injuries were

related to terrorist-linked acts involving structural collapse and the use of toxic material. Previous studies on primary injury (BOP) have traditionally focused on gas-containing hollow organs such as the lungs and gastrointestinal tract (Moore and Jaffee, 2010;Baker et al., 2011).

In one article by Clemedson discussing blast injury, the term "blast injury" has been used to describe the biophysical and pathophysiological events post exposure to high explosion or the shock wave associated with it (Clemedson, 1956). The greatest interest was devoted to study the peak pressure, as well as the impulse relevant to pulmonary injuries produced (Clemedson and Pettersson, 1953;Clemedson, 1954;Celander et al., 1955;Clemedson et al., 1957). Interestingly, on the pathophysiology of blast injury, the sudden alteration in the body ambient pressure causes injury, primarily in gas-air filled organs including the lungs, intestines or in tissues with different specific weight such as the ear and intestines; this occurs at the interface between media with very large differences in density (Clemedson, 1956;Elsayed, 1997;Mayorga, 1997;Guy et al., 2000). Furthermore, BOP can induce a mild form of brain injury with significant neurological conditions involving cerebral edema, neuroinflammation, and vasospasm along with diffuse axonal injury and neuronal death. This neuronal injury phase is followed by a series of complex neuropsychiatric symptoms which may include memory loss and behavioral changes (Okie, 2006;Cernak, 2010;Cernak and Noble-Haeusslein, 2010;Svetlov et al., 2010;Schultz et al., 2011). As such, exposure to complex blast waves can be viewed as the inducer of multitude of injuries or even polytrauma involving several organ injuries interaction that exacerbates blast insult outcome (Schultz et al., 2011). Finally, blast wave propagation to the brain parenchyma is another controversial mechanism which may involves both direct propagation through the skull or in an indirect propagation via blood vessels which has a direct implication on vascular disturbance (This topic has been elegantly evaluated by Cernack et al as discussed later) (Cernak, 2010;Valiyaveettil et al., 2013).

Neuropathological Alteration in Blast injury

Experimental studies of primary blast brain injuries (though limited) have shown evidence of altered cellular, molecular and biochemical processes coupled with altered changes in behavior. For instance, experimental studies have shown a heterogeneous profile of brain-associated cellular impairments including: elevation in β-amyloid precursor protein, altered expression of protooncogenes *c-Myc, c-Fos* and *c-Jun* and impaired axonal transport coupled

with oxidative stress with elevated nitric oxide generation (Saljo et al., 2000;Saljo et al., 2001;Saljo et al., 2002a;Saljo et al., 2002b;Saljo et al., 2003;Ansari et al., 2008;Saljo et al., 2008;Benzinger et al., 2009;Saljo et al., 2009;Svetlov et al., 2009a;Readnower et al., 2010;Svetlov et al., 2010). In addition, neuronal injury and glial activation (discussed later) along with elevation of biochemical markers such as, neuron specific enolase (NSE), ubiquitin C-terminal hydrolase 1 (UCH-L1) and glial fibrillary acidic protein (GFAP) have been reported. Other studies have shown evidence of axonopathy, edema and hypertrophic astrogliosis along with altered gene expression post injury (Cernak et al., 2001;Ansari et al., 2008;DeWitt and Prough, 2009;Readnower et al., 2010). However, there were a lot of ambiguity in the overpressure and duration utilized and the methods used to measure these parameters which were often unclear and not standardized (Svetlov et al., 2009a;Svetlov et al., 2010;Pun et al., 2011).

Furthermore, such heterogeneous neural profile has been attributed to several factors including the suitable experimental model systems that can closely mimic "composite" primary, secondary, tertiary, and quaternary components of blast exposure, the lack of standardized blast wave instruments, different body localization and body armor and the use of different animal species. These factors coupled with others have led to such mixed profile of neural injury (Cernak et al., 1996;Saljo et al., 2008;Cernak, 2010;Cernak and Noble-Haeusslein, 2010).

Several studies have been performed to assess neuropathological effect of blast overpressure coupled with other comorbid factors (Cernak et al., 1996;Mayorga, 1997;Davenport et al., 2011;Kirkman et al., 2011;Koliatsos et al., 2011;Pun et al., 2011;Rafaels et al., 2012). In this section, we will list some of the major blast injury studies where different parameters were varied (different blast injury models, intensity, animal used) or other modifications were included (protective vests, stressors). In their work, Kamnaksh et al assessed different stressors that may contribute to blast traumatic brain injury including transportation and blast sound with or without blast injury. Of interest, all groups exhibited increased anxiety, while injured animals and blast noise exposed rats showed elevated corticosterone, interferon-c (IFN-c) and interleukin-6 (IL-6) in the amygdala and hippocampus. Injured animals showed elevated (Iba1), GFAP and apoptotic immunoreactivity (Kamnaksh et al., 2011). Taken together, these data demonstrate that exposure to biological stressors can lead to behavioral changes and trigger specific neuropathological alteration even in the absence of detectable injury.

Pun et al, using a rat model, assessed the effects of a single sublethal blast over pressure (BOP) exposure (48.9 kPa - 77.3 kPa) in an open-field set up. Microarray platform and histopathological analyses were conducted. Histopathological analysis of inflicted brains revealed "darkened" and shrunken cortical neurons with narrowed vasculature at day 1 post-injury. Signs of recovery were demonstrated at days 4 and day 7 post blast exposure. Oligodendrocytes and astrocytes showed TUNEL-positivity in the white matter at day 1; however, low caspase-3 immunopositivity was observed. Acute axonal damage was observed in the white matter as indicated by elevated amyloid precursor protein immunoreactivity with no sign of macrophages/microglia change. Major gene changes were observed at day 1 and day 4 post-blast pointing toward signs of repair at day 4 and day 7. These findings suggest that the BOP levels in the study resulted in mild cellular injury to the brain as evidenced by acute neuronal, and white matter perturbations that showed signs of repair and resolution (Pun et al., 2011). In another study by Koliatsos et al, primary (BOP) wave effect of mild blast overpressure (68 kPag, 103 kPag and 183 kPag) was compared to secondary and tertiary effects. Using a shock tube generating shock waves, the effects of blast on parenchymatous organs including brain, were evaluated. The main injuries in non-brain organs included hemorrhages in the lung interstitium, hemorrhagic infarcts in liver, spleen, and kidney. Neuropathological changes and behavioral outcomes were evaluated at mild blast intensity showing signs of multifocal axonal injury in the cerebellum, the corticospinal system and optic tract. These findings were accompanied with prolonged behavioral and motor abnormalities (deficits in social recognition, spatial memory and in motor coordination). Interestingly, shielding of the torso ameliorated axonal injury and behavioral deficits (Koliatsos et al., 2011).

In a different study, de Lanerolle et al used a swine model to assess different scenarios of blast exposure including: simulated free field (blast tube), high-mobility multipurpose wheeled vehicle surrogate and building 4-walled structure. Of interest, histological changes in the 3 blast scenarios showed minimal neuronal injury with fiber tract demyelination and intracranial hemorrhage. Neuropathological changes involving increased astrocyte activation coupled with proliferation and periventricular axonal injury detected with β-amyloid precursor protein immunohistochemistry were also observed (de Lanerolle et al., 2011).

Long et al assessed blast-induced physiological, neuropathological, and neurobehavioral changes coupled with Kevlar protective vest encasing the thorax and part of the abdomen. They used a compression-driven shock tube (at 126- and 147-kPa). It was shown that animals with the Kevlar vest reduced air blast mortality, and also ameliorated the widespread fiber degeneration in rat brains. Blast overpressure was shown to induce abnormal neurologic and neurobehavioral performance along with cardiovascular disruptions involving hemorrhagic hypotension, with disruption in cardio-compensatory resilience (reduced peak shed blood volume etc.) (Long et al., 2009). Similarly, Rafaels et al, using a male ferrets whose thorax and abdomen were protected, evaluated intracranial hemorrhage and cardiorespiratory coupling at different ranges of blast exposures. Increasing severity of blast exposure demonstrated increasing apnea immediately after the blast accompanied by hemorrhages in proximity to the brain stem, at the highest blast intensities (Rafaels et al., 2012).

In an interesting study, Garman et al characterized the neuropathological changes produced by a single blast exposure in rats with body shielding using a helium-driven shock tube (exposure of 35 PSI with left side- head-only exposure) (Garman et al., 2011). The insult produced 25% mortality. Neuropathological analysis was conducted at various time points (24 h, 72 h, or 2 weeks post-blast). Multifocal axonal degeneration was present in all blast-exposed rats at all-time points coupled with diffused axonal injury in the cerebellar and brainstem white matter tracts. In addition, multifocal neuronal death at 24 h and 72 h was observed. Reactive microglial activation was also identified despite subtle GFAP, ED1 and Iba1 staining. Finally, increased blood–brain barrier permeability was seen at 24 h and primarily affected the contralateral cortex. Findings from this study indicated axonal, dendritic, neuronal, and synaptic degeneration in the initial 2 weeks in rats with body shielding. Over time, there was also evidence of progression of the axonal degenerative process characterized by increased axonal fragmentation similar to the process of diffuse axonal injury (DAI) that follows TBI which is suggestive of a therapeutic window in the immediate post-blast period (Garman et al., 2011).

In conclusion, these different blast animal model studies are presented with distinguished heterogeneous results; however, they provided different insights into the associated neuropathological changes occurring post blast injury. These findings highlight the challenges encountered in modeling experimental blast injury; this demonstrates that for a successful

translational approach, clinical experience needs to be incorporated into preclinical TBI research and experimental findings should be evaluated/verified clinically (discussed in different sections).

Neuronal Injury Mechanisms:

The exact mechanism by which blast overpressure mediates neuronal injury has not been fully elucidated (Pun et al., 2011). The neuropathological changes evoked by blast overpressure are different than those described following acute models of brain injury ; (i.e. acceleration–deceleration injury or direct impact) (Lighthall, 1988;McIntosh et al., 1989;Dixon et al., 1991;Hall et al., 2008;Long et al., 2009) highlighting at the complex pathways involved. Elegant work with experimental data by Cernak et al has shown that primary closed non-impact blast injury-induced neurotrauma involves the interaction of cerebral, local and systemic responses (Cernak et al., 1996;Cernak et al., 2001;Cernak, 2010;Cernak and Noble-Haeusslein, 2010). These experimental data seem to indicate involves the fact that blood vessels vasculature (venous as well as arterial) may be acting as a conduit for blast energy transfer to the brain contributing to blast pressure -induced fiber degeneration.

In non-blast brain injury, the primary injury occurs as a consequence of mechanical force due to direct contusion of the brain against skull's rough interior or due to shearing and stretching forces against the brain tissue (Greve and Zink, 2009;Cernak, 2010). This may also involve vascular injury including subdural hematoma from ruptured blood, brain edema from elevated permeability of cerebral vasculature along with reduced blood flow due to intracranial pressure or infarction (Greve and Zink, 2009). Taken together, these complications represent the secondary and tertiary phases of blast injury. On the other hand, primary blast surrounds the whole body affecting different organ systems. Its effect on brain can be mediated via a direct or indirect injury. Blast wave passage to the skull causes acceleration/rotation to the brain comprising the direct injury. Indirect injury involves the compression of the abdomen and chest transferring kinetic energy to the body's biofluid. This rippling effect generates oscillating waves from blood to the brain distant from the contact point. In turn, this kinetic energy transfer will induce functional and morphological changes in brain structures which represent a distinct complex feature of blast induced brain injury not present in other traditional brain injury models

(Cernak et al., 1999;Warden et al., 2009;Cernak, 2010). The complex mechanism of blast injury involves consequences of primary blast effects on autonomous nervous system.

Cernack et al assessed the contribution of body-CNS cross talk involved in blast induced trauma related to the activation of autonomous nervous system and the neuroendocrine–immune system which contribute significantly to the molecular changes and blast injury mechanisms. Inflammation has been proposed to play an important role in the pathogenesis of long-term neurological deficits due to blast (Cernak, 2010). Experiments using rigid body- or head protection in animals subjected to blast showed that head protection failed to prevent inflammation in the brain while body protection was able to alleviate blast-induced brain functional impairments highlighting the role of body-CNS interaction (Cernak, 2010).

Cernack et al studies have demonstrated that blast exposure (mild-to-moderate) induces the activation of autonomous nervous system in rabbit model exposed to a blast overpressure generated by a compressed air-driven shock tube. Distinct pathological components in the brain including impaired energy metabolism, and increase in the sodium–potassium ATPase measured in the brainstem and erythrocyte membranes were coupled with edema formation (Cernak et al., 1995;Cernak et al., 1996). In addition, to link systemic alteration and cerebral inflammation to long-term neurological deficits caused by blast, migration and accumulation of polymorphonuclear leukocytes as key inflammatory markers of host response were assessed after helium-driven shock tube delivering mild blast injury (103 kPa) with 5% mortality. *In vivo* real time imaging of myeloperoxidase (MPO) inflammatory enzyme activity of activated phagocytes was conducted on 3 groups of rats (1) whole-body blast; (2) blast with "body armor," (chest and abdomen) with the head exposed or (3) blast with "helmet" as head protection (neck and skull) while the rest of the body exposed. One day post blast exposure, MPO activity was observed in the gastrointestinal tract and the diaphragmal mediastinal parts of the lungs (Cernak et al., 2010).

In the brain, this activity was observed at 7, 14, and 30 days post blast injury. Of interest, MPO increase in the brain was independent of head protection at 14 and 30 days post-injury suggesting chronic inflammation and highlighting the role of systemic origin of the inflammatory activation mediating brain injury which highly reflects on the role of the vagal afferent neurons mediating gut–brain communication. Taken together, the results of this study clearly demonstrate the importance of the indirect, i.e., blast–body interaction as well as the decisive role of

autonomous nervous–neuroendocrine–immune systems interaction in the pathogenesis of blast induced brain trauma (Cernak, 2010).

Similarly, Chavko et al assessed the theory of the indirect effect of kinetic energy transfer via the blood vessels and the surrounding CSF to the CNS (Chavko et al., 2011). In their work, Chavko et al evaluated the contribution of direct versus indirect transfer and its correlation to the head orientation and the surface area exposed to the blast. Brain biomechanical responses involving pressure inside the brains were studied in rats exposed to low blast exposure (35 kPa) and positioned in three different orientations with respect to primary blast wave. These positions included: frontal exposure (i.e. head facing blast) right side exposed to blast and head faces away from blast. Frontal exposures showed higher traces of pressure amplitude and longer duration, suggestive of dynamic pressure transfer (Chavko et al., 2011). On the other hand, the pressure wave inside the brain in the head facing away was similar to hydrodynamic pressure within the brain. It has become more evident that the primary pressure wave can induce functional, biochemical and morphological alterations in different ways than those observed in other types of traumatic injuries, such as those caused by penetrating head injury.

Neuropsychiatric Impairments in Blast Injury and PTSD Comorbidity

Many injured troops returning from war zones are afflicted with blast-induced TBI experiencing post concussive symptoms (PCS), characterized by memory and cognitive disruption, irritability, anxiety, fatigue, and persistent headaches (Okie, 2005). Among these with mTBI, PCS can persist long after exposure leading to major functional impairments (Carroll et al., 2004). Unlike casualties suffered from moderate to severe TBI patients diagnosed with mild TBI (mTBI) present with no apparent structural injury and are conscious with typical symptoms including headache, confusion, dizziness, memory impairment, and behavioral changes. mTBI is the most frequent form of brain trauma among deployed military populations (Vanderploeg et al., 2012). It has been shown that repeated exposure to multiple low levels of blast injury account for the majority of mTBIs cases. These victims remain conscious and often are redeployed without proper diagnosis and treatment while they undergo severe mental stress (Trudeau et al., 1998;Santiago et al., 2010). The heterogeneous presentation of blast overpressure injuries among mTBI patients depends on several factors (similar to what is observed in experimental blast

injury studies) including: device composition, environment (e.g., presence of intervening protective barriers), distance from blast and the use of protective shields (Kelvar vests etc.) (DePalma et al., 2005;Taber et al., 2006).

It has been shown that primary blast component of blast injury is among the main contributors in developing neuropsychiatric impairments associated with the primary phase profile (Guy et al., 2000;Kocsis and Tessler, 2009). There had been an urgent quest to for future research examining the impact of blast concussion (particularly recurrent concussion) on neuropsychological performance. Neuropsychological evaluation of cognitive status post blast exposure can be challenging for a variety of reasons. In particular, clinicians may have difficulty assessing: true concussion severity due to limited knowledge of the blast events which may be reflective of self-report months or years after the event(s). In addition, the lack of several features of the blast environment may complicate the accuracy of the "blast self-report" involving distance from the blast, concussion severity which these are often unavailable from primary records (Nelson et al., 2011). Thus, the lack of reliable information pertaining to injury characteristics makes it challenging to determine the course of cognitive recovery and rehabilitation. Usually, concussion severity is usually determined based on current post concussive symptoms on screening instruments which are not necessarily specific to concussion and can be shared with depression or PTSD or even these PCS may be reflective of PTSD itself as elegantly discussed by Nelson et al (Nelson et al., 2011). Of interest, Hoge et al reported that more than 40% of soldiers who experienced symptoms associated with mild TBI (loss of consciousness) met the criteria for PTSD (Hoge et al., 2008). This same study suggested that increased rates of health problems reported by soldiers exposed to mild TBI are mediated mainly via neuropsychiatric disorders such as PTSD or depression, rather than mild TBI (Hoge et al., 2008).

PTSD, a psychiatric condition that arises after exposure to a life threatening experience such as conditions experienced in combat war zone with or without blast exposure as a form of mild TBI (Zatzick et al., 2010). This, by itself, poses a challenge in the clinical diagnosis in veterans who are exposed to mTBI since the symptoms may overlap between these conditions exacerbated by other comorbid conditions such as drug abuse or other neuropsychiatric complications (Zatzick et al., 2010;Seal et al., 2011). A Rand Corporation study indicated that

approximately 20% of returning service personnel (~300,000) have had a TBI and that there was substantial overlap of TBI with the occurrence of PTSD (Tanielian and Jaycox., 2008). This poses a major challenge to the military health care personnel. Psychological stress resulting from exposure to blast wave exposure leads to an altered psychological health status which contribute significantly to the development of PTSD (Trudeau et al., 1998;Kamnaksh et al., 2011). However, a major recurring question arises-due to the similarity of blast injury clinical symptoms and TBI with those of PTSD-, is how do we clinically differentiate between these two conditions and from other neuropsychiatric conditions?

For neurological assessment in TBI, similar criterion-based methodology to that in PTSD has been used rendering a specific diagnosis to either condition or even to those with both conditions (PTSD and TBI-exposed) uncertain (First et al., 1995;Bombardier et al., 2006;Bass et al., 2011). Thus, in many cases, clinical diagnosis may result in high rate of inaccurate PTSD in persons exposed to TBI (Bass et al., 2011). This is exacerbated by the fact that military epidemiology studies rely on patient's self-reported information about blast occurrence which result in a falsely diagnosis of PTSD condition which may actually represent TBI or vice versa (Bass et al., 2011).

Among these non-specific symptoms are lack of concentration, insomnia and irritability, which are difficult to specifically attribute to either PTSD or mTBI. An accurate detailed knowledge of blast injury biophysics and injury threshold may assist clinicians in better diagnosis (Bass et al., 2011). This includes expanded neuropsychological studies of blast injury (both experimental and clinical) to identify accurate, specific and sensitive "anatomic, pathophysiologic, and behavioral responses to blast injury as discussed by Bass et al (Bass et al., 2011). Several factors contribute to inaccurately diagnosing blast injury which may be complicated by the complex nature of blast injury involving several combinations of primary or other phases of blast injury (secondary, tertiary, and/or quaternary blast).

Clinical evaluation of a blast exposed personnel can be challenging as symptoms may range from neurologic problems, psychiatric or emotional difficulties which may be attributed to blast or due to other psychiatric disorder where in several instances the occurrence of TBI and PTSD may be suggested (Rosenfeld and Ford, 2010;Bass et al., 2011). For instance, there are a number of symptoms of impaired concentration, increased irritability insomnia, and lack of

interest that are shared in the diagnosis for mild TBI and PTSD (APA, 2000). These overlapping symptoms described leaves us with major challenges in understanding the exact pathophysiology of blast injury pertaining to standardize assessment of injury severity for mild TBI in both humans and animal models, and finally, understanding the relationship between clinical manifestation of PTSD, mild TBI and acute TBI (Bass et al., 2011).

Animal Models of Blast Injury

Over the last several decades, a number of experimental animal models have been implemented to study the mechanisms of blast wave impact which included rats, mice, ferrets, rabbits and larger animals such as sheep and swine (Irwin et al., 1998;Garner et al., 2009;Svetlov et al.;Svetlov et al., 2010;Rubovitch et al., 2011;Lei et al., 2012;Li et al., 2012;Sundaramurthy et al., 2012;Yarnell et al., 2013). These experimental models showed heterogeneous results and even contradictory findings which have been attributed to several factors. A summary of the recent and major blast injury studies (2001, 2009-2013) is summarized in **Table 1**. In addition, there is a lack in the reproducibility of blast injury models and a need to develop blast injury generators that precisely control of the blast injury parameters similar to other well-defined acute brain injury models such as (controlled cortical impact (CCI) and the fluid percussion (FP) which have been well characterized with predictable neurological, histological, and physiological changes similar to those observed in clinical acute brain injury settings. Thus, the need of establishing a well characterized reproducible experimental model (animal and blast framework) is vital to identify relevant pathogenic pathways involved that can help in the development of blast specific-biomarkers or panel of biomarkers. Furthermore, There are still major gaps in the understanding of the complex pathophysiological mechanisms involved in blast injury which reflect highly on the challenge for the discovery of the accurate and effective diagnostic, prognostic markers as well as therapeutic tools (Kochanek et al., 2009). Several blast injury instrumentations are available which include: compressed gas-driven shock tubes which are driven by air, helium or nitrogen gas which may result in unrealistic duration of the overpressure wave leading to an inappropriate scaling between species (humans and animal models).

Challenges in Animal Models of Blast Injury

There are limited available basic and translational studies relevant to the mechanisms of primary blast-induced brain injury. A better understanding of injury mechanisms is required for the development of protection and treatment options and biomarker identification for prognosis. Several animal models have been proposed at translating intra-cranial biophysics and pathophysiology experienced in human blast exposure (Bass et al., 2011). However, these models have a number of limitations inherent to the different animal models used including: neuronal tissue biomechanical properties, anatomical differences as well as physiological differences (Bass et al., 2011). In addition, other factors that are challenging for proper scaling between experimental and human blast injury are associated with neuroanatomy and physiology involving: size of different brain structures, neural mass (brain size, head, body, position and architecture), as well as body fluid composition (thickness, volume, and components) (Bass et al., 2011). Other key factors that need to be considered when designing animal blast injury models are the potential for exposure scaling, consistency in experimental protocols, frequency of exposure and overpressure levels, which should be mimicking real life exposure or at least translate equally to human exposure (**Figure 1**). Other external factors include: distance from the blast, the use of protective shields and the presence or absence of noise stressors etc. (Belanger et al., 2011) (**Figure 1**). In real life situation, soldiers are often deployed several times and many are exposed to numerous psychological stressors such as blast noise with or without blast injury (Bass et al., 2011). Such conditions can induce adverse physiological changes leading to post-traumatic symptoms without sustaining any prior physical injury (discussed previously). Taken together, several challenging factors exist that contribute to the difficulty of truly modeling blast injury in animals resulting in an in-appropriate neuropathological and neurobehavioral assessment.

Blood Brain Barrier and Secondary Injury in Blast Overpressure

Traumatic brain injury (TBI) leads to progressive pathophysiological changes resulting in a reduction in brain blood flow and a decrease in tissue oxygen levels leading to ischemia, subsequent secondary injury, blood brain barrier breakdown and brain edema (Unterberg et al., 2004). Death of resident cells of the central nervous system (CNS) has traditionally been accepted to take place in two phases: an early necrotic and an on-going long-term apoptotic

phase. Secondary brain injury develops in minutes to months following the original insult, progressively contributing to the worsened neurological impairment. This complex phenomenon is defined by the activation of various neurochemical cascades and the systemic physiological responses which manifest following the traumatic event (Morganti-Kossmann et al., 2007).

At the cellular level, the biphasic nature of secondary injury is mediated by numerous disturbed pathways which include: (a) excitotoxicity caused by an excess of the neurotransmitter glutamate; (b) free radical generation by mitochondrial dysfunction, causing damage to proteins and phospholipid membranes of neurons and glia; and (c) the neuroinflammatory response which takes place due to both CNS and systemic immunoactivation. Moreover, microglial activation within injured loci was observed within 6–48 hours post-injury. Thus, diffuse brain injury mediated immune responses, blood–brain barrier (BBB) alterations and neuroinflammation seem to play an important role in the pathology. The increase in BBB permeability was shown to recover by the third day after the blast exposure (Readnower et al., 2010;Abdul-Muneer et al., 2013). There are several factors, i.e. inflammatory mediators, free radicals, proteases, adhesion molecules, VEGF, bradykinin and arachidonate metabolites, that enhance edema formation and BBB dysfunction (Unterberg et al., 2004). Following blast injury, loosening of the vasculature and perivascular unit was mediated by the activation of matrix metalloproteinases and fluid channel aquaporin-4, promoting edema, enhanced leakiness of the BBB and progression of neuroinflammation and neuronal degeneration (Abdul-Muneer et al., 2013). Although many studies demonstrate a similar pathophysiologic progression as the conventional TBI, a recent study reported that cerebrovascular injury due to primary blast is distinct from it; suggesting that BBB disruption in blast injury was an acute one, not resulting from a delayed inflammation as it is in the conventional ones (Yeoh et al., 2013).

Recent work from our laboratory has shown that blast injury leads to oxidative stress and autonomic dysfunction (Tumer et al., 2013a). Generation of free radicals and hypoxia leads to the failure of the Na^+, K^+-ATPase, a membrane-bound enzyme required for cellular transport. Dysfunction of this pump is a common feature in CNS pathologies related to ischemic conditions and TBI. The activity of Na^+-K^+-ATPase pump is very sensitive to free radical reactions and lipid peroxidation. Reductions in this activity can indicate membrane damage indirectly. Thus Na^+, K^+-ATPase is clearly down regulated under low O_2 conditions which in turn triggers brain

edema, enhances the loosening of tight junctions and causes BBB breakdown. Myeloperoxidase activity, an index for neutrophil infiltration, also increases as an evidence of inflammation (Biber et al., 2009). In summary, failure of pumps, cerebral edema, BBB permeability, neuroinflammation and oxidative damage are among the major mechanisms that play important roles in the development of secondary brain injury following TBI.

Traumatic Brain Injury and Autonomic Dysfunction

One deleterious consequence of brain injury is autonomic nervous system dysregulation and/or dysautonomia. Autonomic nervous system dysfunction has been documented after traumatic brain injury but is not well understood. Ninety percent of traumatic brain injury patients demonstrate signs of autonomic dysfunction during the first week after injury, with about one third of the patients developing longer lasting autonomic dysfunction. Autonomic dysregulation is characterized by distinct changes in cardiovascular hyperactivity, sleep function and specific biomarkers of neural damage. System dysregulation might lead to a range of comorbidities such as hypertension, endothelial dysfunction, and end-organ perfusion abnormalities. Specifically, TBI disruption of autonomic function most often results in sustained sympatho-activation. This sympathetic hyperactivity after traumatic brain injury remains poorly understood, although sympathetic hyperactivity likely contributes to the high morbidity and mortality associated with TBI. Sympathetic hyperactivity contributes to systemic stress, including neuroinflammation and oxidative stress in the autonomic nervous system. Eventually these disturbances lead to cardiovascular dysfunction (Cernak, 2010;Cernak and Noble-Haeusslein, 2010;Hinson and Sheth, 2012) and sleep complications (Viola-Saltzman and Watson, 2012). Systemic stress is associated with activation of the hypothalamic-pituitary-adrenal (HPA) axis (Griesbach, 2011) and the hypothalamic sympatho-adrenal medullary axis (Kvetnansky et al., 2009). It is known that traumatic brain injury activates the HPA, however little is known regarding the traumatic brain injury -induced activation of the sympatho-adrenal medullary axis, and there are limited therapeutic options to treat this sympatho-activation.

We recently demonstrated selective biochemical markers of autonomic function and oxidative stress in male Sprague Dawley rats subjected to head-directed overpressure insult (Tumer et al., 2013a). There were increased levels of tyrosine hydroxylase (TH), dopamine-β

hydroxylase (DβH), Neuropeptide Y (NPY) along with plasma norepinephrine (NE). In addition, blast-induced injury significantly elevated TH in the nucleus tractus solitarius (NTS) of the brain stem while AT1 receptor expression and NADPH oxidase activity, a marker of oxidative stress, was elevated in the hypothalamus suggesting that single BOP exposure results in increased sympatho-excitation. The mechanism may involve the elevated AT1 receptor expression and NADPH oxidase levels in the hypothalamus. Taken together, such effects may be important factors contributing to pathology of brain injury and autonomic dysfunction associated with the clinical profile of patients following BOP exposure (Tumer et al., 2013a).

Blast brain Injury and Oxidative Stress

The primary effects of BOP have been generally attributed to its external physical impact on the body, causing internal mechanical damage. The pathophysiological effects on hollow organs have been extensively studied, but little attention has been given to the biochemical manifestations and molecular mechanism(s) of injury occurring in the brain after BOP exposure. Due to the biochemical nature of BOP compared to physical nature of traumatic brain injury (impact or penetrating injury), subtle molecular changes such as free radical-mediated oxidative stress occur and contribute to the manifestation of BOP-induced brain injury (Ansari et al., 2008;Readnower et al., 2010;Kochanek et al., 2012). Previous studies have demonstrated that reactive oxygen species such as the superoxide radicals and nitric oxide can form peroxynitrite, a powerful oxidant that impairs cerebral vascular function following blast induced brain injury (Vuceljic et al., 2006;DeWitt and Prough, 2009). Cernak et al, reported that bilateral vagotomy successfully mitigated bradycardia, hypotension, and apnea caused by blast; prevented extreme metabolic alterations and brain edema; but failed to eliminate oxidative stress in the brain due to blast (Cernak et al., 1996). More recently, it was reported that the induction of oxidative and nitrosative damage leads to cerebrovascular inflammation in an animal model of mild traumatic brain injury induced by primary blast (Abdul-Muneer et al., 2013). Brain-specific oxidatively modified protein markers that are indicative of biochemical/proteomic and functional changes occurring post-BOP need to be considered. Insufficient published data are available to describe the long-term effects of TBI on central noradrenergic systems, particularly on neuroplastic adaptations within numerous targets of central noradrenergic projections. In addition,

understanding the etiology of these changes may shed new light on the molecular mechanism(s) of injury, potentially offering new strategies for treatment.

Blast Injury and Biomarkers Identification

The widespread recognition of the brain vulnerability to blast exposure and inadequate approaches to diagnose blast-related TBI led to design a mTBI Diagnostics Workshop (Marion et al., 2011) and the foundation of the Demographics and Clinical Assessment Working Group of the International and Interagency Initiative (Menon et al., 2010) to assess the current diagnostics technologies that can be used to detect brain injury following mild TBI and blast exposure. One of the major recommendations was the use of biomarkers to supplement functional and imaging-based assessments for significant improvements in the diagnosis and characterization of the effects of blast exposure on brain and for distinguishing bTBI from other neuropsychiatric disorders including PTSD.

Current available imaging modalities, such as computed tomography (CT) and magnetic resonance imaging (MRI), primarily detect major structural changes in the brain (Bazarian et al., 2006); however, their utility has not been fully optimized following blast-related mild TBI. More advanced neuroimaging techniques such as DTI, while have showed abnormalities post blast-related TBI (Mac Donald et al., 2011), have not been able to show consistent relationship to mild bTBI diagnosis (Levin et al., 2010). Additionally, there is no consensus on the ideal scan method or timing. Therefore, multiple studies have been conducted to identify ideal sensitive, inexpensive, noninvasive biochemical markers that can offer diagnostic and prognostic information, and reflect bTBI pathogenic mechanisms and pathology (Mondello et al., 2011;Mondello et al., 2013).

To date, several biomarkers such as GFAP (Papa et al., 2012a) UCH-L1 (Papa et al., 2012b) and S-100ß (Unden and Romner, 2010) have been identified as potential excellent "candidates" of blast TBI. However, a limited number of studies did specifically evaluate biochemical brain damage markers in the setting of blast-induced brain injury (Agoston et al., 2009;Svetlov et al., 2009b). One prominent targeted biomarker study was conducted by Svetlov et al. In their study, they assessed temporal pattern of serum putative biomarkers that have been characterized in acute TBI including glial fibrillary acid protein (GFAP), neuron specific

enolase, and ubiquitin C-terminal hydrolase (UCH)-L1 in brain tissue, cerebrospinal fluid (CSF), and blood after 10 msec of 358 kPa peak overpressure blast exposure. Serum biomarkers levels distinctively increased 24 hours post blast, followed by a decline thereafter, indicating a potential use to assess blast-induced brain damage acutely after injury. (Svetlov et al., 2010). Supporting these observations, Gyorgy and colleagues, using reverse phase protein microarray (RPPM) technology to determine serum protein levels, showed a rise in S100B, MBP, NF-H and NSE protein levels in serum after injury in a large-animal model of bTBI. Remarkably, serum NF-H was reported to increase in an overpressure dose-dependent manner reflecting the extent of the damage caused by bTBI (Gyorgy et al., 2011).

More recently, Balakathiresan et al proposed microRNAs as novel serum diagnostic biomarkers of bTBI. They investigated the microRNA signatures in CSF and serum of rats exposed to blast overpressure injury. Specifically, microRNA let-7i elevated in both CSF and serum post blast wave exposure was identified as an ideal candidate biomarker of TBI (Balakathiresan et al., 2012). Importantly, microRNAs can be considered the third generation molecular signature after proteomics and genomics studies (Balakathiresan et al., 2012). Elevated concentrations of serum vascular endothelial growth factor, associated with neuroinflammation and vascular pathology in blast-related TBI have also been reported (Agoston and Elsayed, 2012).

Studies investigating biomarkers of mTBI in humans continue to be limited as illustrated in one study by Ingebrigtsena and Romnerb (Ingebrigtsen and Romner, 2003). In their research paper, MEDLINE database was surveyed for biochemical serum markers specific to mild head injuries. Of interest, three serum markers creatine kinase isoenzyme BB (CKBB), neuron specific enolase (NSE) and S-100B were evaluated. Of these markers, S-100B protein was proposed as the most promising marker for mTBI while the other two lacked specificity, sensitivity or injury correlation (Ingebrigtsen and Romner, 2003). In an another study by Blennow et al, military personnel exposed to explosions or repeated firing of heavy weapons did not show any evidence of brain damage as assessed by CSF biomarkers. (Blennow et al., 2011). Conversely, the New Zealand Breacher Study demonstrated a degree of brain perturbation as assessed by serum biomarker levels, neurocognitive performance and self-reported symptoms in members of the New Zealand Defense Force exposed to repeated low-level blast (Tate et al.,

2013). Taken the controversial results of these different studies, these findings, in fact, stimulate the need for further research to evaluate the usefulness of biochemical markers after repeated exposure of different blast levels.

Interestingly, recent experimental and human studies are suggesting a link between blast exposure and chronic traumatic encephalopathy (CTE), a tau protein–linked neurodegenerative disease (Omalu et al., 2011;Goldstein et al., 2012;Miller, 2012;Huber et al., 2013). To date, no biofluid marker has been shown to assist with diagnosis of CTE. However, future studies to identify biomarkers tracking chronic processes and ongoing degeneration and able to predict the development of neurodegenerative diseases of bTBI are of a critical need.

An important consideration is that a panel combining different biomarkers be achieved which can establish the nature and severity of the head injury and reflect at the contributing pathogenic mechanism(s) of the acute phase as well as the neurodegeneration and recovery (rehabilitative stages). Additionally, the integration of such bTBI diagnostic markers into routine clinical care will require a thorough validation and extensive standardization at different validation levels as well as well-defined recommendations for immunoassay and different measurement technologies.

A non-trivial and urgent issue in biomarker-panel design will be determining an appropriate instrument platform that is suited to measure these biomarker changes. At present, biomarkers are analyzed in clinical laboratories using closed, high throughput immunoassay analyzers allowing for high performance in terms of accuracy and precision which are suitable for major hospitals. Future recommendation is to focus research on the development of a miniaturized point-of-care (POC) system, which can be transported in the 'field' (military and civilian) providing accurate measurements at a reasonable cost with short turnaround time (Mondello et al., 2011).

Conclusion

For long, TBI has been considered 'signature injuries' of current conflicts in Iraq and Afghanistan which attracted concern from the DoD, Department of Veteran Affairs and National Institutes of Health (NIH), encouraging combined efforts to understand brain injury pathophysiology and identify therapeutics and assess different approaches for rehabilitation

platforms as well as deciphering novel blast specific biomarkers (DePalma et al., 2005;DePalma et al., 2011). Better understanding of the biophysics of blast shock injury and its body propagation to the neural tissue may enhance the development body armor protection. Given the complexity of blast TBI pathobiology, the development of an objective specific quantifiable panel of biomarkers is highly needed for the purpose of providing better depiction of the real time injury mechanism and progression post blast exposure (Berger, 2006;Beers et al., 2007;Agoston et al., 2009;Gyorgy et al., 2011).

Figure Legends

Figure1: Challenges Associated with "Experimental Blast Injury" Modeling Real Life Blast Exposure. Several factors contribute to the heterogeneous behavioral, neuropathological and systemic profile observed in the several experimental blast injury models. Even with models using the same injury parameters (animal model, blast shock tube and intensity levels etc.); reproducing the same results is challenging (refer to Table 1). These challenging variables are summarized in the following: (A) various animal models and interspecies variation, (B) blast injury frequency and intensity levels ranging from single blast up to 5 blast with some overpressure intensities reaching 515 kPa (C) the heterogeneous selection of biochemical/behavioral testing conducted and the several time points selected [hrs. to few months] (D)the non-standardized blast and not well characterized blast injury instrumentation (E)technical variation inherent to experimental design related to animal setting, body armor, head protection, and the distance from the blast. These factors contribute to the variable outcome observed in published work in blast injury field.

Table1 : Recent Major Studies on Experimental Blast Injury withe Different Parameters Assessed (Behavioral, Neuropathological and Biomarker changes).

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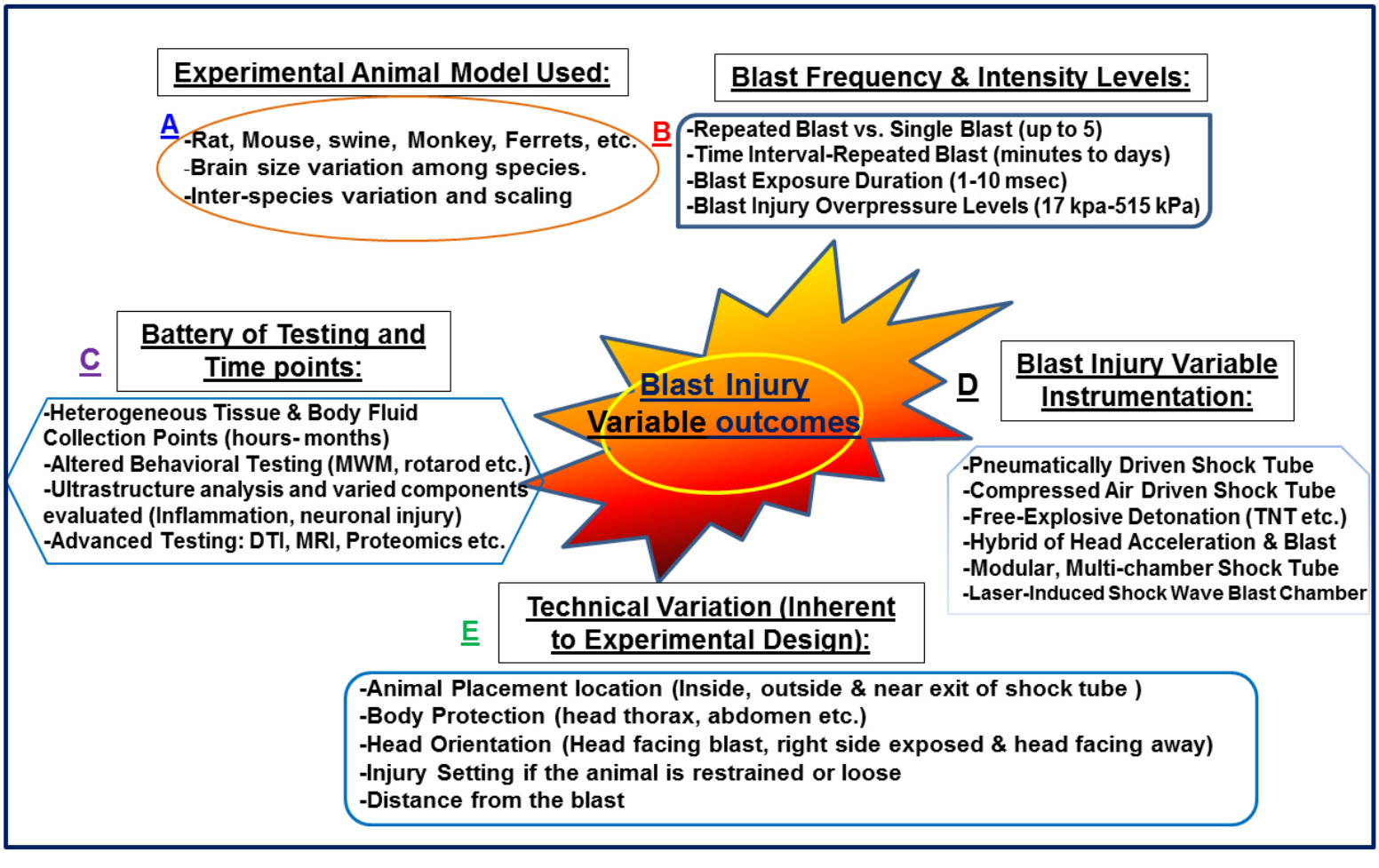
Figure 1.JPEG



Anxiety Produced in Rats by Over-Pressurization Blast Injury

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ABSTRACT

Traumatic brain injury (TBI) occurs in individuals exposed to direct force trauma and close proximity explosions. Blast TBI is an injury that is the result of an explosion generating an over-pressurization blast (OB) wave in close proximity to the individual producing closed-head brain injury. Individuals experiencing Blast TBI have behavioral (post-traumatic stress disorder, PTSD), respiratory (apnea) and cardiovascular changes directly correlated with the blast-induced TBI. There is also a correlation between a history of TBI and PTSD. PTSD symptoms include anxiety and fear. An OB injury rat model was used in this study to test the hypothesis that anxiety/fear can be induced in rats following exposure to a moderate to severe OB. The elevated-plus maze (EPM) test was used before and after OB to assess anxiety. Rats underwent EPM testing 4-5 days prior to the OB. The animals were anesthetized and the OB brain injury produced by a compressed air-driven shock tube directed at the dorsal surface of the head of the rat with an average peak overpressure of 90 psi at the shock-tube resulting in a 52 psi at head with a total duration of 10 msec of blast exposure. The body was protected from the pressure wave by a plexiglass cover. Three days following the OB, the rats were retested on EPM. Animals spent 60.9% of time in closed arm pre-OB and 89.9% of time in closed arm post-OB ($p < 0.05$). Animals spent 30.9% of time in the center pre-OB and 0.10% of time post-OB ($p < 0.05$). Animals spent significantly shorter time in open arm post-OB ($p < 0.05$). There were no significant differences in these EPM parameters for control animals. These results would suggest that rodents exposed to a moderate to severe blast wave have heightened anxiety/fear possibly associated with blast-induced TBI.

INTRODUCTION

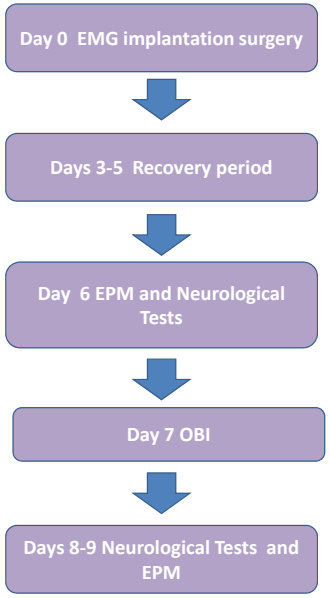
Mild traumatic brain injury affects nearly two million people yearly at an estimated annual cost of $76.5 billion in the United States (Brain Injury Association of America). The leading cause of TBI are motor-vehicle crashes, falls and sporting accidents, violence and firearms in the civilian population. Whereas military personnel suffer the majority of TBIs from blast injuries caused by improvised explosive devices (IEDs) (Brain Injury Association of America). TBI causes behavioral as well as physiological changes. Depression following TBI is highly variable and ranges from 6% to 77% of TBI patients (Morton and Wehman 1995). Male rats exposed to repetitive blasts experienced PTSD symptoms including increased anxiety (Elder, et al., 2012). An impact acceleration model of TBI demonstrated long-term increase in stress and anxiety in rats consistent with depression (Fromm, et al., 2004).

Experimental blast injury induces a traumatic brain injury by directing a jet of pressurized air at the skull. This pressurized air sends an impulse wave throughout the brain and brainstem causing shearing and stressing of the tissue and potentially disrupting neuronal connections. Concussion models have shown excitotoxicity that affect the hippocampus and other brain areas controlling affective behavior following a TBI. Our animals are exposed to an over-pressurization blast injury (OBI) used to model blast TBI. Diaphragm EMG activity was used to determine the effect of OBI on breathing pattern and the elevated plus maze (EPM) was used to test fear and anxiety.

HYPOTHESES

1. We hypothesized that OBI would induce an initial apnea followed by variable and decreased ventilation.
2. We hypothesized in conscious rats exposed to OBI there would be increased fear/anxiety.

METHODS



Animal Model
•Adult male Sprague Dawley rats (n=27) weighing between 250 to 310 grams were anesthetized using Isoflurane.
•Diaphragm EMG electrodes were implanted. The animals were allowed to recover and Dia EMG activity recorded pre-OBI to ascertain a reliable EMG signal.
•Animals were transported to laboratory where the OBI was induced.
• Animals were anesthetized with Isoflurane. OB brain injury was produced by a compressed air-driven shock tube directed at the dorsal surface of the head of the rat near Bregma. The pressure wave was delivered with an average peak overpressure of 90 psi at the shock-tube resulting in a 52 psi at head with a total duration of 10 msec of blast exposure. The body was protected from the pressure wave by a plexiglass cover.
•Diaphragm EMG was recorded in the anesthetized rat 5 minutes prior, during and 5 minutes post-OBI using PowerLab and LabChart software. The EMG was integrated with a 50 msec moving time average.

Behavioral Testing
Conscious animals received behavioral tests prior and after OBI.
EPM: The animal was placed in the center of an EPM. Rat position and movement were recorded to determine the time spent in open, closed and middle sections.
Proprioception and Vibrasse: A rod was used to elicit a withdrawal response by tapping the skin in the external oblique area (left and right) and brushing caudal to cranial through the vibrasse. The withdrawal response was scored on a 1-3 scale: 1=no response, 2=response one side and 3=response both sides

Statistical Analysis
•A paired t-test between groups was used to determine significance in pre- and post-blast measurements as well as irregular breathing and psi levels.
•A one-way repeated measure analysis of variance with Shapiro Wilk normality test between groups was used to determine significance in apneic and irregular ventilton periods.
•The significance criterion for all analyses was set at p<0.05.

RESULTS

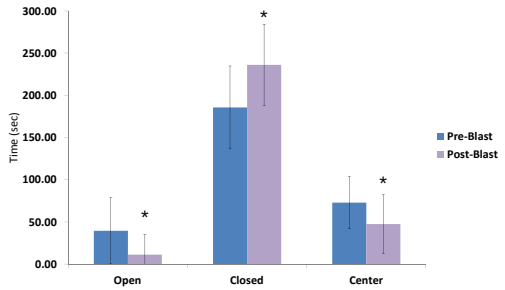


Figure 1. Elevated Plus Maze. The animals were tested pre- and post-blast. They spent less time in both the open and center sections of the elevated plus maze and more time in the closed section. All sections reached statistical significance (*$p < 0.05$).

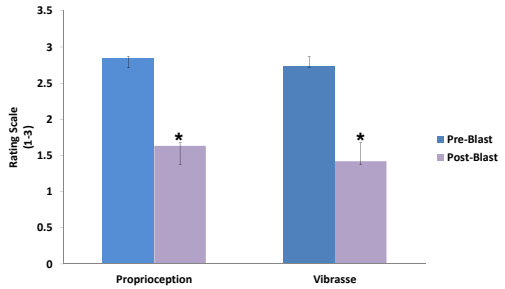


Figure 2. Neurological Testing. Proprioception and vibrasse withdrawal responses significantly decreased 24 hours post-blast. *($p < 0.05$).

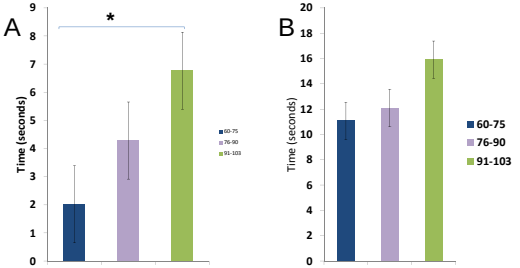


Figure 3. A. OBI Pressure and Apneic Period. Between 60-75 psi and 91-103 psi statistical significance was seen for apneic period (sec). No significance was seen between 76-90 psi and either 60-75 psi or 91-103 psi. * $p < 0.05$). **B. OBI Pressure and Irregular Breathing Period.** Irregular breathing is defined as augmented or attenuated inspiratory efforts until return to a post-blast baseline breathing pattern. No significance was seen in groups. *($p < 0.05$).

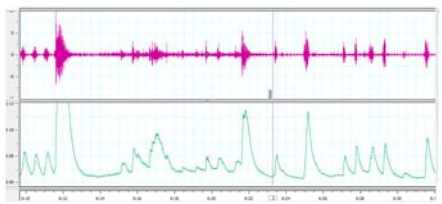


Figure 5. Animal 5, psi 102.6. Irregular breathing pattern following OBI apnea. Panel A: Raw dEMG signal. Panel B: Integrated dEMG signal.

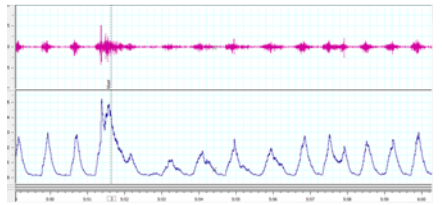


Figure 6. Animal 19, psi 69.9. Irregular breathing pattern following OBI apnea. Panel A: Raw dEMG signal. Panel B: Integrated dEMG signal.

CONCLUSIONS

- OBI induced apnea and irregular ventilation during pressure exposure in anesthetized rats.
- The apneic periods were significantly longer in the 91-103 psi group when compared to the 60-75 psi group. This is consistent with animal studies that show increased apneic period in higher severity brain injuries.
- The apneic period was not significantly different between 76-90 psi group and either 60-75 psi or 91-103 psi groups suggesting a threshold for OBI related apnea.
- Apnea is different as a function of psi but the duration of disordered breathing was similar in all animals.
- The blast injury resulted in a decrease in time spent in the open arms of the EMP consistent with OBI induced increase in fear/anxiety response.
- The OBI resulted in decreased somatosensory function evidenced by decreased sensation to proprioception and vibrasse stimuli as a result of brain injury.

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